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SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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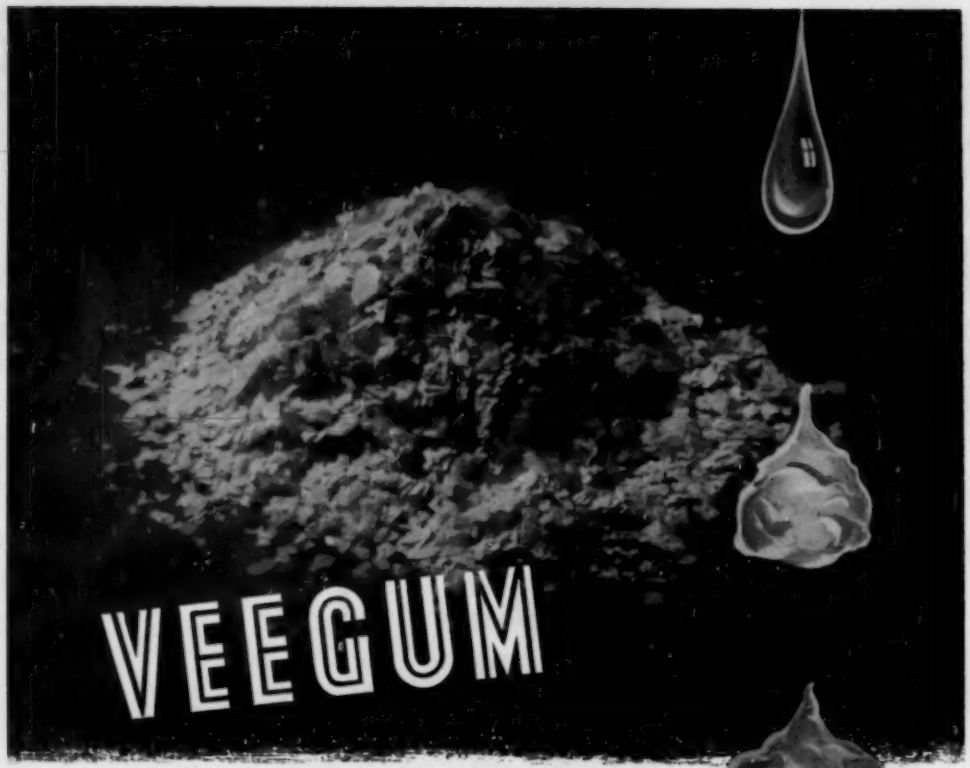

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STATEMENT OF INCOME AND EXPENSE (GENERAL FUND)

Income			
Sale of Pharmacopeias			
Collections		$22,699.30	
Deduct: Decrease in Accounts Receivable			
Balance, December 31, 1953	$ 7,728.70		
Balance, December 31, 1954	3,634.30	4,094.40	$18,604.90
Sale of Reference Standards			
Collections		17,618.35	
Add: Increase in Accounts Receivable:			
Balance, December 31, 1954	1,740.64		
Balance, December 31, 1953	857.20	883.44	
		18,501.79	
Add: Adjustment of Prior Year's Collections		336.10	18,837.89
Interest on Investments		8,052.50	
Less: Amortization of Bond Premiums		22.65	8,029.85
Interest on Savings Deposits			2,608.19
Profit on Sale of Investments			6,562.86
Miscellaneous			275.67
Total Income			54,919.36
Less Expense			
Revision			
Cash Disbursements		63,445.96	
Less: Increase in Inventory			
Balance, December 31, 1954	15,696.66		
Balance, December 31, 1953	14,607.63	1,089.03	62,356.93
Administration			4,741.67
Publications			
Cash Disbursements		4,274.95	
Add: Decrease in Inventory			
Balance, December 31, 1953	2,352.24		
Balance, December 31, 1954	332.00	2,020.24	6,295.19
Headquarters			
Cash Disbursements		4,142.52	
Add: Depreciation Expense			
Building	2,420.77		
Furnishings and Equipment	3,133.32	5,564.09	9,705.61
Total Expense			83,100.40
Excess of Expense over Income			$28,181.04

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

Cash on Deposit, December 31, 1953					$120,953.56	
Add: Receipts						
Sale of Pharmacopeias				$22,699.30		
Sale of Reference Standards				17,618.35		
Interest on Investments				9,452.50		
Interest on Savings Deposits				2,333.20		
Sale of Investments				54,978.89		
Miscellaneous				275.67	107,357.91	$228,311.47
Less: Disbursements						
Expense:	Revision	Administration	Publications	Headquarters	Total	
Printing and Binding	$	$	$4,274.95	$	$ 4,274.95	
Salaries	37,865.32	4,115.00	....	1,307.00	33,287.32	
Meetings	2,782.67	98.39	....	....	2,881.06	
Supplies	1,421.16	....	....	....	1,421.16	
Postage and Telegrams	1,786.36	40.00	....	....	1,826.44	
Utilities	....	....	....	1,801.34	1,801.34	
Repairs and Maintenance	....	....	....	884.18	884.18	
General	19,590.45[a]	488.20	....	....	20,078.65	
Taxes	....	....	....	150.00	150.00	
Furnishings and Equipment	426.85	....	....	....	426.85	
	$63,872.81	$4,741.67	$4,274.95	$4,142.52	$77,031.95	77,031.95
Cash on Deposit, December 31, 1954						$151,279.52

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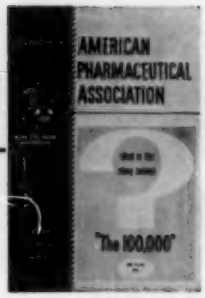


A Preview of Advertising

In the May 1955 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the porducts mentioned.)

▶ In two insertions, **Abbott Laboratories** publicizes its antibiotic, *filmtab* **Erythrocin Stearate,** and new *iron-plus* **Iberol.**

▶ Advocated for varicose vein complications, thrombophlebitis and bursitis is **My-B-Den,** product of the Bischoff Division of the **Ames Company.**

▶ **Span, Tween, Sorbo** and **Arlacel** are some of the pharmaceutical adjuncts offered by the **Atlas Powder Company.**

▶ **Thephorin Lotion** is pointed up by **Hoffman-La Roche** for treating poison ivy, insect bites and miscellaneous summer itches.

▶ The confirmed value of **Mercurochrome** as an antiseptic is emphasized by **Hynson, Westcott & Dunning.**

▶ **Achromycin S.F.** is **Lederle's** broad-spectrum antibiotic with the stress vitamin formula suggested by the National Research Council. Also offered by the same company is a new anticholinergic, **Pathilon.**

▶ **'Prenalac'**—product of **Eli Lilly and Company**—is described as providing the "recommended daily vitamin and mineral allowances. . ."

▶ Counseling the maintenance of adequate stocks, the manufacturer catalogues the following **Massengill** prescription specialties: **Adrenosem, Aminodrox, Gallogen, Livitamin, Massengill Powder, Obedrin, Salcort** and **Semhyten.**

▶ Emphasized by **Owens-Illinois** in its insertion is the visibility of new **Duraglas Clear Dry Rx Squares.**

▶ On stage for **Parke, Davis** are **Combex Kapseals,** prescribed when high-potency vitamin B complex and vitamin C are needed.

▶ **Pfizer's** antibiotics, **Terramycin, Tetracyn** and **Pen,** are each tagged "SF"—Stress Fortified.

▶ **The Philadelphia College of Pharmacy and Science** is aptly described as the "alma mater of successful pharmacists."

▶ Prescribed for peptic ulcer is **Robalate**—for local therapy only, and **Donnalate**—for local plus systemic therapy—products of **A. H. Robins Company.**

▶ Currently promoted by **Roerig** are **Vi-Thyro,** "the new *vitalized* thyroid," and **Am Plus,** "for safe obesity control."

▶ "When hormones are preferred therapy . . . **Schering** hormones," states their producer, "assure superior quality."

▶ For use in the treatment of diarrhea, **Sharp & Dohme** calls attention to **Cremosuxidine.**

▶ The ready saleability of **Benzedrex Inhalers**—in their well publicized display carton—is reemphasized by **Smith, Kline & French Laboratories.**

▶ **Mysteclin,** offered by **Squibb,** is "antibacterial therapy plus antifungal prophylaxis in one capsule."

▶ The **Upjohn Company's** announcement realistically implies that in the treatment of diarrhea **Kaopectate** is indicated as a matter of course.

▶ New sizes and forms of **Peritrate,** its prophylaxis against angina pectoris, and **Methium,** its hypotensive agent, are reflected in the **Warner-Chilcott** announcement.

▶ "New pharmaceutical elegance for a standard sedative"—**Luminal Ovoids**—product of **Winthrop-Stearns.**

Look for details in the Practical Pharmacy Edition of This Journal—Out May 20

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIV — *MAY, 1955* — NUMBER 5 — CONSECUTIVE NO. 10

Metal Chelates and Antitubercular Activity

I. *o*-Hydroxyazo Naphthols and Phenanthrols*

By WILLIAM O. FOYE and J. GEORGE JEFFREY†

A series of iron, copper, and chromium chelates of some *o*-hydroxyazo naphthols and phenanthrols has been found to suppress the growth of *M. tuberculosis* H37Rv both *in vitro* and *in vivo*. While the *in vivo* activity, as measured by survival times of mice, is relatively weak, it is distinctly different from that of the nonmetallized compounds. Spectral and analytical evidence has indicated a 1:1 ratio of metal to azo compound in these chelates with all three metals, and general structures are postulated.

EVIDENCE has been provided in a number of recent reports to suggest that the antitubercular action of various agents is due to their ability to complex heavy metal ions, or that these agents are active as metal complexes. Carl and Marquardt (1), for instance, have suggested a direct quantitative relation between the antitubercular activities of such drugs as *p*-aminosalicylic acid, the benzal thiosemicarbazones, and isonicotinyl hydrazide and their abilities to form complexes with cupric ion. Liebermeister (2) also showed that a number of antitubercular thiourea, thiouracil, and thiosemicarbazone derivatives formed cupric complexes, and found that several of these complexes were active *in vitro* against mycobacteria. Roth, Zuber, Sorkin, and Erlenmeyer (3) later tested the cupric complex of *p*-aminosalicylic acid and found approximately the same antitubercular activity *in vitro* as *p*-aminosalicylic acid itself, both being increased by excess cupric ion. Bonicke and Reif (4), however, differed from the original postulation of Carl and Marquardt (1), and found no relation between the *in vitro* antitubercular activities of a large number of *p*-aminosalicylic acid derivatives and their metal-complexing abilities.

Erlenmeyer's group also found that the antitubercular action of 8-hydroxyquinoline was increased significantly in the presence of cupric ion (5), but reduced by cobaltous and molybdate ion (6). Similar results were also obtained with a number of other phenolic and enolic compounds, which generally showed increased *in vitro* activity in the presence of cupric and decreased activity with cobaltous ion (7). Albert, Gibson, and Rubbo (8), after observing the activity of 8-hydroxyquinoline against Gram-positive organisms, however, concluded that the metal-oxine complexes were the true toxic agents and the activity was not due to an ability to complex metal ions. Their tests were carried out with cupric, ferrous, and ferric ions.

A series of metal chelates of some azo naphthols and phenanthrols previously prepared in this

* Received August 27, 1954, from the School of Pharmacy, University of Wisconsin, Madison.

Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.

Abstracted from a portion of the dissertation presented to the Graduate School of the University of Wisconsin by J. G. Jeffrey in partial fulfillment of the requirements for the degree of Doctor of Philosophy, October, 1954.

† On leave of absence from the School of Pharmacy, University of Saskatchewan, Saskatoon, 1953–1954.

The authors are extremely grateful to Drs. K. K. Chen, C. G. Culbertson, W. B. Sutton, and P. N. Harris of the Lilly Research Laboratories for carrying out the antitubercular and antitumor determinations and providing the results. Appreciation is also expressed to the Canadian Foundation for the Advancement of Pharmacy for financial assistance.

TABLE I.—ANTITUBERCULAR ACTIVITIES OF THE AZO NAPHTHOL AND PHENANTHROL METAL CHELATES

Metal Chelate[a]	*In Vitro* Activity[b]	Survival Time of Mice in Days — Chelate	Drug[c]	Controls
OAP → α-Naphthol, Fe	−	18	19.8	17.3
OAP → β-Naphthol, Cr	−	11	11.7	8.6
OAP → β-Naphthol, Cu	−	10.6	18.0	17.0
1-A-2-N → β-Naphthol, Cr	−	16.3	18.3	15.6
1-A-2-N → β-Naphthol, Cu	−	9	11.7	8.6
OAP → 1-Phenanthrol, Cr	−	16.4	18.3	15.6
OAP → 1-Phenanthrol, Fe	−	16	18.3	15.6
OAP → 2-Phenanthrol, Cr	−	17.3	20.6	17.5
OAP → 2-Phenanthrol, Cu	++	15.5	20.6	17.5
OAP → 2-Phenanthrol, Fe	−	18.6	20.6	17.5
OAP → 3-Phenanthrol, Cr	−	17	20.6	17.5
OAP → 3-Phenanthrol, Fe	−	17	19.8	17.3
OAP → 4-Phenanthrol, Cr	+++	17	18.3	15.6
OAP → 4-Phenanthrol, Fe	++	10	11.7	8.6
Controls	+++			

[a] OAP = o-aminophenol, 1-A-2-N = 1-amino-2-naphthol → = azo linkage.
[b] A minus sign indicates inhibition of growth.
[c] Either streptomycin or p-aminosalicylic acid was used as comparison drug.

laboratory (9) has been tested for antitubercular activity both *in vitro* and *in vivo*. The results are stated in Table I. While complete inhibition of *M. tuberculosis* H37Rv was general for these compounds *in vitro*, their activities when tested in mice were relatively weak. These activities are based on the survival times of infected mice when fed the metal chelates, compared with both the survival times of mice treated with *p*-aminosalicylic acid or streptomycin and the survival times of the control animals. However, the *in vivo* activities of the metal chelates differ significantly from those of the unmetallized azo compounds, as shown in Table II. Here, the two azo

TABLE II.—ANTITUBERCULAR ACTIVITIES OF UNMETALLIZED AZO NAPHTHOLS

Azo Naphthol[a]	*In Vitro* Activity[b]	Survival Time of Mice in Days — Naphthol	Drug[c]	Controls
OAP → α-Naphthol	−	12	19	17.1
OAP → β-Naphthol	−	18.4	23.3	17.5

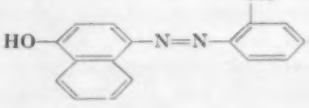
[a] OAP = o-aminophenol, → = azo linkage.
[b] A minus sign indicates inhibition of growth.
[c] Either streptomycin or p-aminosalicylic acid was used as comparison drug.

compounds available in the greatest quantity and highest state of purity were tested. The α-naphthol derivative was found to be quite toxic, while its iron chelate showed very slight activity. The β-naphthol derivative, however, showed questionable activity, but its chromium chelate showed relatively good activity and its copper chelate was highly toxic.

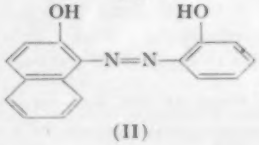
These results indicate that the metal chelates are active as such, although the possibility still exists that exchange reactions with other metal ions might occur *in vivo*. Since these compounds were administered to the infected mice in the diet, it is also evident that absorption of the intact chelate molecule occurs, despite their relative insolubility and tendency to decompose in acid media.

STRUCTURAL CONSIDERATIONS

The structures of the different types of azo compounds listed in Table I are represented by the following four compounds: 4-(*o*-hydroxyphenylazo)-1-naphthol (I), 1-(*o*-hydroxyphenylazo)-2-naphthol (II), 1-(*o*-hydroxyphenylazo)-2-phenanthrol (III), and 1-(2′-hydroxy-1′-naphthylazo)-2-naphthol (IV). Azo compounds of this type can exist either in phenolic or keto forms, according to the evidence of Kuhn and Jär (10). By determining the absorption spectra of the two phenylazo naphthols and their O- and N-methyl derivatives in various solvents, they concluded that 4-phenylazo-1-naphthol existed mainly in the phenol form in neutral solvents and 1-phenylazo-2-naphthol existed mainly in the keto form in either neutral, basic or acidic media.



(I)



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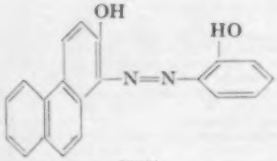
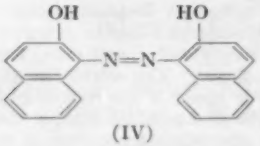
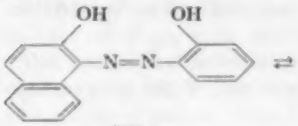


(III)

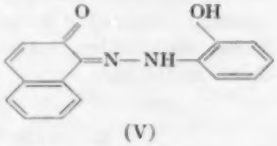


(IV)

The absorption spectra of the *o*-hydroxyphenylazo naphthols were obtained in this investigation, and the curves corresponded closely to those of Kuhn and Bär. Whereas Kuhn reported single maxima at 410 mμ and 475 mμ, respectively, for 4-phenylazo-1-naphthol and 1-phenylazo-2-naphthol, we found single maxima at 420 mμ and 485 mμ for the corresponding *o*-hydroxyphenylazo napththols in acetone. Structure (II), then, should more correctly be represented in the keto form (V):



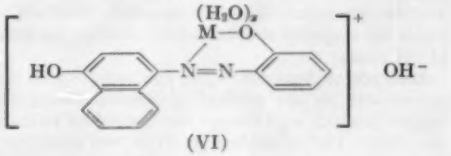
(II)



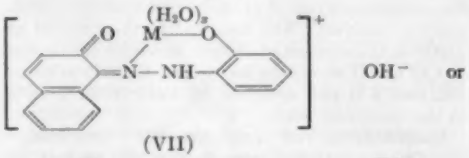
(V)

The structures of the metal chelates of azo compounds have not been established with certainty, particularly the chromium complexes. A 1:1 ratio of metal to azo compound is generally indicated, however. Grimmel (11), in his review of azo dyes, states that in the case of chromium chelates, different forms containing different ratios of chromium to dye are known. Both Krzikalla (12) and Schetty (13), however, postulate structures containing a 2:3 ratio of chromium to azo dye. Analysis of the *o*-hydroxyphenylazo naphthol metal chelates, the results of which are shown in Table III, although somewhat inexact, indicates a 1:1 ratio of metal to azo compound with either chromium III, copper II, or iron II. The exact nature of the chelates is difficult to picture, however, because of the varying amounts of water of hydration that result on drying the compounds (14) and because of the possibility of acetoxy groups still attached to chromium in the chromium chelates. Ferrous chelates also give unsatisfactory analyses due to air oxidation to ferric iron and simultaneous formation of the oxide. In any case, excess metal in the form of salt or hydroxide is difficult to remove from the metal chelates.

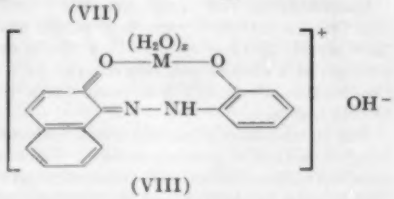
The structures of the metal chelates of the *o*-hydroxyazo naphthols can most likely be represented by the general formulas VI–VIII, on the basis of the analytical results and absorption spectra, as well as previous work (11).



(VI)



(VII)



(VIII)

Scrutiny of the Fisher-Taylor-Hirschfelder models of structures (VII) and (VIII) showed either one capable of existence. It was impossible to form a structure having valence bonds between the metal and both oxygens simultaneously with a nitrogen-to-metal coordination, however.

Four of the metal chelates, those appearing first, second, third, and fifth in Table I, were also tested for tumor inhibition against sarcoma 180 in mice. Daily intraperitoneal doses of these complexes had no appreciable effect in nontoxic amounts.

TABLE III.—ANALYSES OF METALLIZED AZO NAPHTHOLS

Azo Naphthol	Formula[a]	Calculated	Found[b]
OAP → β-Naphthol, Cu	$C_{16}H_{11}N_2O_2 \cdot Cu \cdot OH$	C, 55.89	C, 55.13
		H, 3.52	H, 3.16
		Cu, 18.48	Cu, 21.9
OAP → β-Naphthol, Cr	$C_{16}H_{11}N_2O_2 \cdot Cr \cdot 2OH$	C, 55.01	C, 55.27
		H, 3.75	H, 4.16
		Cr, 14.89	Cr, 12.5
OAP → α-Naphthol, Fe	$C_{16}H_{11}N_2O_2 \cdot Fe \cdot OH \cdot 4H_2O$	C, 47.08	C, 47.49
		H, 4.69	H, 3.49
		Fe, 15.77	Fe, 18.3
OAP → α-Naphthol, Cr	$C_{16}H_{11}N_2O_2 \cdot Cr \cdot 2OH \cdot 2H_2O$	C, 49.87	C, 50.81
		H, 4.44	H, 4.42
		Cr, 14.90	Cr, 17.1

[a] The complexes were dried *in vacuo* over $Mg(ClO_4)_2$ before C—H analysis. Small but variable losses of weight were observed, so the products analyzed contain less than the theoretical amounts of water of hydration. More thorough drying before the metal analyses caused greater dehydration with the α-naphthols.

[b] The copper and iron analyses were carried out by the Wisconsin Alumni Research Foundation, Madison. The analyses for carbon-hydrogen and chromium were carried out by the Clark Microanalytical Laboratory, Urbana, Ill.

EXPERIMENTAL

Purification of the Metal Chelates.—The metal chelates of the azo naphthols and phenanthrols, after preparation as described in a previous report (9), were washed with water and dried. After being ground to fine powder, they were extracted with Skellysolve® B in a Soxhlet extractor fitted with a fritted-glass thimble until no more color was removed from the product. This required twenty-four to forty-eight hours. The resulting powders gave negative tests for sulfur (ferrous sulfate, copper sulfate, and chromium acetate were used to make the chelates) after sodium fusion, and negative tests for sodium with the zinc-uranyl acetate reagent. When suspended in distilled water the majority showed faintly alkaline reactions to *p*H paper.

Methods of Analysis.—The analysis for iron was carried out by the method of Sandell (15). The copper analysis was carried out according to Skinner (16). The chromium analysis was done from the ash remaining after a carbon-hydrogen combustion analysis. The ash was further heated at 1100° in the absence of oxygen to assure conversion to Cr_2O_3. It should be mentioned that these metal analyses are not accurate to more than 5–10% in the quantities used.

Determination of Antitubercular Activities.—The *in vitro* activities were determined against *M. tuberculosis* H37Rv in modified Proskauer-Beck medium at a chelate concentration of 0.02 mg./ml. Observation of growth was made for a period of twenty-one days.

The *in vivo* determinations were made with mice infected with the same organism. Generally five mice were observed for each concentration of metal chelate used over periods of thirteen to twenty-one days. The chelates were administered in the diet in concentrations of 0.02–0.5%. The survival times stated in Table I represent an average of the survival times found with the different concentrations of chelate, excluding those where the amount of chelate was great enough to be toxic or small enough to exert no effect. The drugs used for comparison were either streptomycin, 500 units daily administered subcutaneously, or *p*-aminosalicylic acid, 1% in the diet. Eight to ten mice were observed with the comparison drug. In the case of the control animals, given no agent, generally ten mice were observed.

The per cent mortalities of the mice fed the metal chelates were usually intermediate to those for mice fed the comparison drug and to those of the controls.

Absorption Spectra Determinations.—The Cary Recording Spectrophotometer using visible light (Tungsten source and Corex cells) from 800–275 mμ was employed. The solvent in all cases was acetone. The speed of the recording paper corresponded to a change of 25 mμ per division, thus giving only approximate positions of the maxima. The concentrations of the azo compounds were: 4-(*o*-hydroxyphenylazo)-1-naphthol, 0.02 Gm./L.; 1-(*o*-hydroxyphenylazo)-2-naphthol, 0.01 Gm./L. The maxima observed for these compounds were at approximately 420 mμ and 485 mμ, respectively.

SUMMARY

1. Antitubercular activity, both *in vitro* and *in vivo*, has been found for a series of metal chelates of some azo naphthol and phenanthrol derivatives.

2. The metal chelates show a significant difference in activity from that of the nonmetallized azo compounds.

3. Analytical evidence indicates these complexes to have a 1:1 ratio of metal to azo compound, with either iron II, copper II, or chromium III.

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Metal Chelates of Streptomycin*

By WILLIAM O. FOYE, WINTHROP E. LANGE, JOSEPH V. SWINTOSKY, ROBERT E. CHAMBERLAIN, and JOSEPH R. GUARINI

Streptomycin has been found to undergo chelation with several metals in aqueous solution. Evidence for chelate formation is provided by a drop in *p*H during the formation, production of characteristic colors, decreased solubility in water, and absence of metal ions in the solution. The copper chelate, found to be relatively uncontaminated with metal hydroxide, exhibited more prolonged blood levels in guinea pigs than streptomycin sulfate, but also a greater toxicity.

THE NUMEROUS centers for chelation present in the streptomycin molecule make metal chelation of streptomycin in solution highly probable. The possibility then exists that metal chelates of streptomycin might provide a form of the antibiotic from which stable suspensions could be prepared, and which might also provide a longer-acting preparation than those at present available. Accordingly, streptomycin was reacted with a number of metal salts capable of forming chelates, and several metal chelates of lower solubility in water than streptomycin itself have been isolated. Previously, methods of preparing stable solutions of streptomycin have been described, using calcium chloride (1) and such agents as thioglycerol, thiosorbitol, and thioglucose (2), but no stable, insoluble preparations have been reported.

CHELATE FORMATION

The preparation of the metal chelates was found to proceed in either aqueous or aqueous-alcoholic solutions, similar to the preparation of metal chelates of riboflavin already reported by Foye and Lange (3). Aqueous solutions of the metal salts were added to the streptomycin solutions in equimolar quantities along with sufficient alkali to maintain a *p*H of 9, which was found to favor chelate rather than metal hydroxide formation in most cases. The drop in *p*H which occurred without addition of alkali during the reaction also provided evidence for chelate formation rather than salt formation. In general, the chelates precipitated from solution either at once or after chilling.

Chelation of streptomycin was attempted with divalent copper, nickel, cobalt, iron, magnesium, and zinc. The most successful reactions for producing chelates of lower water solubility than streptomycin sulfate were those carried out with copper, nickel, and cobalt. Magnesium gave a water-soluble chelate, as might be expected with alkaline earth metals (4), ferrous iron yielded an unstable product contaminated with excessive amounts of the metal hydroxide, and no evidence for chelation with zinc could be discovered. The physical properties of the chelates prepared for biological testing are listed in Table I.

TABLE I.—STREPTOMYCIN-METAL CHELATES

Metal Chelate	Color	Decomposition Temperature
Streptomycin-copper	Pale blue	165–170°
Streptomycin-nickel	Green	160–165°
Streptomycin-cobalt	Blue-green	145–150°
Dihydrostreptomycin-copper	Dark blue	178–183°

Analyses.—Analyses for carbon, hydrogen, and metal were obtained for the chelates in an attempt to determine the ratio of metal atoms to streptomycin. The results are considered inconclusive because of the unknown purity of the commercial streptomycin (Merck and Co., Inc.) used, because the numerous centers for chelation in the streptomycin molecule make it impossible to calculate the amount of water of hydration filling the unused coordination capacities of the metal atoms, and because the only means of purification found possible for these chelates was washing with cold water. However, both the carbon-hydrogen and metal analyses for the copper chelate indicate a ratio of three copper atoms to one streptomycin molecule. The nickel and cobalt chelates were found to contain appreciable amounts of metal hydroxide, as indicated by positive tests for metal ion, which explains the low values found for carbon and hydrogen with these products.

That products of a definite composition were obtained was indicated by varying the ratio of metal salt to streptomycin in the reaction. The small differences in the analytical results of products formed from one, two, or three moles of metal salt to one mole of streptomycin are attributed to contamination by metal hydroxide, and they are not considered great enough to change the ratio of metal to streptomycin in the products. Increasing the molar ratio of nickel nitrate from one to three, for instance, reduced the carbon content from 14.34 to 11.13% and the hydrogen content from 3.82 to 3.37%. Varying the ratio of cobaltous chloride from one to two moles changed the carbon analysis from 7.14 to 5.40% and the hydrogen analysis from 3.08 to 3.04%.

Chelate Formation Criteria.—Martell and Calvin (5) have described a number of criteria for determining chelate formation, among which the following were shown by the streptomycin preparations: color of product, decreased aqueous solubility, drop in *p*H during formation, and absence of metal

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ions in solution after chelate formation in the case of copper. The presence or absence of metal ion was determined by bringing the reaction mixture to a *p*H of 5 to 7 and adding ferricyanide ion or *α*-nitroso-*β*-naphthol as indicator. No color change resulted with the copper chelate, but faint colors were obtained with the nickel and cobalt chelates. When the *p*H was adjusted below 5, however, and the chelates were decomposed, marked color changes were noted. In addition, the product from reaction with ferrous ion, which had an exceptionally high metal analysis, darkened and showed evidence of decomposition on standing. None of the other preparations showed this instability either as the dry solid or as a suspension in ethylene glycol after six months in screw-capped vials.

Dihydrostreptomycin was also reacted with several metal salts, and evidence for chelation was again observed. These products were more soluble in water than the streptomycin chelates, although good yields could be obtained from aqueous-alcohol solutions. Only the copper chelate was prepared in quantity for biological testing, however. The carbon-hydrogen and metal analyses did not agree for a definite ratio of metal to dihydrostreptomycin.

TABLE II.—STREPTOMYCIN POTENCY OF THE STREPTOMYCIN-METAL CHELATES

Metal Chelate	Theoretical Potency (μg./mg.)	Actual Potency (μg./mg.)[a]
Streptomycin-copper	625	354
Streptomycin-nickel	655	<40
Streptomycin-cobalt	646	61
Dihydrostreptomycin-copper	640	240

[a] Samples of each complex were diluted and assayed in two different solvent systems: (1) *p*H 8 phosphate buffer; (2) acetic acid + water + *p*H 8 phosphate buffer by a standard plate assay procedure.

BIOLOGICAL RESULTS

Determinations were made by a standard plate assay procedure to compare the antibiotic potency of the metal chelates with that of streptomycin itself. The samples were assayed in phosphate buffers at *p*H 8, and the results are shown in Table II, where the theoretical potency is based on the streptomycin content of the chelates. Controls run with copper ion alone showed no activity.

A comparison of the durations of activity of the chelates and streptomycin sulfate was made by determining blood levels of these compounds in guinea pigs at intervals. Nine guinea pigs each were injected subcutaneously with equivalent amounts (as determined by the above assay) of streptomycin-copper chelate suspensions and streptomycin sulfate solutions in glycerol-water. Blood samples were withdrawn at the stated intervals, pooled, and assayed for streptomycin activity. The comparison is shown in Table III.

While it is evident that the copper chelate produced a longer-acting product than streptomycin sulfate, it was also more toxic. Of the nine animals injected with the complex, two died within a few hours and two others developed abscesses within two or three days after injections.

TABLE III.—AVERAGE BLOOD LEVELS IN GUINEA PIGS OF STREPTOMYCIN-COPPER AND STREPTOMYCIN SULFATE

No. of Hours after Injection[a]	Blood Levels: Streptomycin-copper	Blood Levels: Streptomycin sulfate
0	0	0
3	1.5	1.6
5	0.9	0.3
7	0.8	0
11	0.3	0

[a] Nine male guinea pigs were injected subcutaneously with streptomycin-copper (5000 μg./Kg.) in glycerol-water vehicle. A second set of nine guinea pigs was given an equivalent amount of streptomycin sulfate in the same vehicle.

Tests for fungistatic activity were also carried out for these chelates, and only the cobalt chelate showed any activity. Slight inhibition was observed against five of the seven pathogenic fungi submitted to the test.

EXPERIMENTAL

Streptomycin-Copper Chelate.—The preparation of the copper chelate of streptomycin, which showed no contamination with metal hydroxide, will be described as representative of the metal chelations. A solution of 1.0 Gm. (0.0014 mole) of streptomycin sulfate (Merck and Co., Inc.) in 10 ml. of distilled water was brought to a *p*H of 9 by addition of 5% sodium hydroxide solution. An aqueous solution (5 ml.) of 0.24 Gm. (0.0014 mole) of cupric chloride was added slowly (five minutes) to the stirred solution along with sufficient 5% alkali to maintain the *p*H at 9. After thirty minutes of stirring at room temperature, the reaction mixture was chilled and filtered. The residue was washed with several portions of ice water and dried *in vacuo*. A yield of 0.90 Gm. of pale blue product was obtained (75% based on a ratio of three atoms of copper to one molecule of streptomycin).

Anal.[1]—Calcd. for $C_{21}H_{34}O_{12}N_7Cu_3 \cdot 6H_2O$: C, 28.73; H, 5.16; Cu, 21.85. Found: C, 28.99; H, 4.90; Cu, 19.77.

From the same molar quantities of streptomycin sulfate and nickel nitrate, 0.50 Gm. of product was obtained.

Anal.—Found: C, 14.34; H, 3.82; Ni, 30.84.

Using cobaltous chloride in an equimolar ratio with streptomycin sulfate, 0.35 Gm. of product was obtained.

Anal.—Found: C, 7.14; H, 3.08; Co, 48.53.

Dihydrostreptomycin-Copper Chelate.—This procedure illustrates the use of an aqueous-alcohol medium. A solution of 0.5 Gm. (0.0007 mole) of dihydrostreptomycin sulfate (Merck and Co., Inc.) in 10 ml. of distilled water was brought to a *p*H of 9 by addition of 5% sodium hydroxide solution.

[1] The carbon-hydrogen analysis was carried out by the Clark Microanalytical Laboratory, Urbana, Ill. The copper analysis was conducted according to the procedure of Hillebrand, W. F., and Lundell, G. E. F., "Applied Inorganic Analysis," John Wiley & Sons, Inc., New York, 1929, p. 199.

The nickel and cobalt analyses were determined from the ash resulting from the carbon-hydrogen analyses, assuming the formulas NiO and CoO, since consistent values could not be obtained from the methods described in Hillebrand and Lundell, *loc. cit.*, p. 316 and p. 326, respectively.

At this point sufficient 95% ethanol was added slowly until the solution contained 40% ethanol, higher concentrations of alcohol result in gum formation. An aqueous solution (10 ml.) of 0.24 Gm. (0.0014 mole) of cupric chloride was added slowly (ten minutes) to the stirred solution along with sufficient 5% alkali to maintain the *p*H at 9. After thirty minutes of stirring, the product was isolated as described in the preceding procedure. A yield of 0.70 Gm. of dark blue product was obtained.

Anal.—Found: C, 12.28; H, 3.71; Cu, 35.69.

Properties of the Chelates.—The metal chelates of streptomycin were found to be slightly soluble in water, ethanol, diethyl ether, and glacial acetic acid; insoluble in propylene glycol, and aqueous CMC; and completely soluble in acetic acid-water solutions. They are stable in boiling water, and at *p*H values above 5, but are decomposed by mineral acids below a *p*H of 5. They showed decomposition temperatures at 150° or above. Elemental analysis showed traces of sulfur and of sodium.

The products showed no evidence of decomposition on storage in screw-capped vials after six months' observation. When suspended in ethylene glycol in screw-capped vials, again no decomposition was observed, although the suspensions settled after several weeks.

SUMMARY

1. Evidence is presented for the formation of metal chelates of streptomycin with copper, nickel, and cobalt. The copper chelate alone showed no evidence of contamination by metal hydroxide. The chelates showed no evidence of instability above a *p*H of 5.

2. The copper chelate of streptomycin was found to provide a more prolonged blood level in guinea pigs than streptomycin sulfate, but its usefulness is limited by a greater toxicity.

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The Effect of Shelf Life on the Potency of Complex Multivitamin Preparations*

By J. A. CAMPBELL and H. A. McLEOD

Samples of different lots of ten complex multivitamin products, formulated as capsules, tablets, and liquids, were purchased on the retail market in Canada and analyzed for their vitamin content. The potencies of vitamin A, vitamin B_{12}, thiamine, and pantothenic acid were markedly affected by shelf life and storage conditions particularly in certain types of products. Riboflavin, niacinamide, pyridoxine, and ascorbic acid contents were less influenced by these factors. Over one-third of the products purchased at random were more than one year old and a significant loss of potency in several products was evident at eight to twelve months after manufacture. This situation indicated a serious lack of adequate control of the potency and marketing of such products and a need for closer cooperation between manufacturer, wholesaler, and pharmacist. The use of an expiration date based on actual stability was suggested as an aid in ensuring that the consumer obtain full labeled potency. It would also be mutually helpful to both the conscientious manufacturer and pharmacist.

ORAL MULTIVITAMIN PRODUCTS, as a class, undoubtedly constitute the largest use of vitamins in the pharmaceutical industry. It is important that these products meet labeled claim during their "normal" shelf life. Because of the instability of many vitamins and their incompatibility with other ingredients, the longer a vitamin product remains on a drugstore shelf, the greater is the possibility that the potency of the vitamins will be below the labeled claim.

Many examples of incompatibility between individual pairs of vitamins may be found in the literature and need not be reviewed here. Several workers have investigated the potency of relatively simple mixtures of vitamins. Siemers (1) discussed the general subject of stability particularly in relation to injectable products. Riboflavin, niacinamide, and pyridoxine were found to be quite stable, while thiamine and ascorbic acid showed considerable loss. Panthenol appeared to be more stable than calcium *d*-pantothenate. Vitamin A stability was better in soft gelatin capsules than in other oral products.

Prindle (2), using accelerated storage tests, showed that loss of vitamin A was related to the complexity of the formula. He pointed out that

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such tests can be related to shelf life only when substantiated by room temperature data. He noted that some effects can be observed only in room temperature storage.

In a study of simple multivitamin liquid preparations, Stone (3) showed that thiamine, niacinamide, and ascorbic acid experienced a marked drop in potency when stored for one year at room temperature. Riboflavin had a tendency to precipitate out of the solutions used. Taub, *et al.* (4), studied the stability of thiamine hydrochloride and thiamine mononitrate in parenteral injections containing riboflavin and niacinamide along with different iron salts. The mononitrate showed slightly better stability in the presence of ferrous gluconate and under nitrogen. Bird and Shelton (5) and Parkington and Waterhouse (6) found that thiamine salts differed somewhat in their stability, depending on the type of preparation used. Since this work was completed, Blitz, *et al.* (7), reported that vitamin B_{12} in the presence of thiamine and niacinamide was quite unstable. In Great Britain Bagnall and Stock (8) carried out a comprehensive survey of the vitamin A content of market samples of halibut liver oil. They pointed out the importance of shelf life and storage conditions and concluded that a date stamp would be necessary to ensure the maintenance of the guaranteed potency of these products.

It is evident from the work reported that stability of vitamins varies with the type of product and that there is still much to be learned about this problem. Mixtures studied have been relatively simple and in many cases have not represented those currently available on the retail market. As Prindle (2) has pointed out, accelerated storage trials may not reflect actual shelf life under certain conditions. Earlier work in this laboratory (9) indicated that vitamin preparations purchased directly from the manufacturer varied markedly in potency depending on whether or not they were produced under the supervision of an adequate control laboratory. More recent work has suggested that, even under what may be considered the best of control facilities, some complex mixtures are relatively incompatible. It is the purpose of this paper to present analytical data on representative multivitamin products in the form of tablets, capsules, and liquids and to discuss factors affecting the potency of these products during their normal shelf life in retail outlets.

PROCEDURE

Ten multivitamin products distributed by six of the larger Canadian manufacturers were selected for this study. Three of these were capsules, four were tablets, and three were liquids. A Food and Drugs Inspector was instructed to purchase at random on the retail market, several samples of each of three or four lot numbers of each product. In some cases it was not possible to obtain this number of lots, in others it was impossible to obtain several samples of each lot. A total of seventy-one samples was obtained for analysis as shown in Table I. Data on the age of products were based on eighty-four samples. Data are also presented on one vitamin A and D capsule.

The vitamin analyses were carried out usually within two months after purchase. In general, because of the volume of work involved, duplicate analyses were carried out only if a product was significantly below label claim.

Vitamin A was determined by the spectrophotometric procedure utilizing the Morton and Stubbs correction as outlined by U. S. P. XIV (10). Thiamine was converted to thiochrome and estimated fluorometrically (11) and riboflavin was also assayed fluorometrically (11). Pyridoxine was determined colorimetrically by coupling with 2,6-dichloroquinonechloroimide (11, 12). Niacinamide was hydrolyzed to niacin and then reacted with cyanogen bromide in the presence of ammonia (13). Depending on the interfering substances that were present, ascorbic acid was estimated by either the indophenol titration (10), the colorimetric procedure of Roe, *et al.* (14), or the recent colorimetric reaction of Schmall, *et al.* (15). Where any difficulty was encountered, ascorbic acid was determined by two methods.

Calcium *d*-pantothenate was estimated microbiologically using *L. arabinosus* 17–5 (16) and vitamin B_{12} using *L. leichmannii* A. T. C. C. 7830 (17, 18).

The date of manufacture of each lot was obtained through the cooperation of the various manufacturers concerned.

RESULTS

Table II lists the constituents of the various products used in this study. Some contained minerals in addition to vitamins.

Potency of Products.—The analytical data obtained for each sample of each product are presented graphically in Fig. 1. The vitamin content found was expressed as a percentage of labeled potency and the samples were arranged in order of date of manufacture as judged by lot numbers.

The lack of stability of vitamin A is particularly noticeable in liquids. All three liquid preparations and one tablet were far below labeled claim and showed wide fluctuations between lots. Variation in the capsules tested was much less but there was a tendency for them to be slightly below labeled claim.

The stability of thiamine in capsules and tablets was satisfactory but in two liquids the thiamine content was variable and often far below labeled potency.

Riboflavin and niacinamide values were relatively stable in all samples, although in liquid J wider fluctuations above and below labeled potency occurred in riboflavin. Two products, capsule A and tablet G, seemed very slightly but uniformly below labeled claim in riboflavin. The majority of samples showed a satisfactory niacinamide content.

Pyridoxine and ascorbic acid were relatively stable, although some samples of tablets D and F fell well below labeled potency and showed wide variations in pyridoxine content. Only tablet F appeared to be consistently below claim in ascorbic acid.

Calcium *d*-pantothenate and vitamin B_{12} exhibited relatively poor stability. Some samples of capsules A and C were well over 50% below labeled potency for pantothenic acid, while capsule B was satisfactory. Liquid J fluctuated widely in potency. Vitamin B_{12} was present in only one capsule (A) and showed good stability. All samples of liquid I and tablets E and G were well below labeled potency in this vitamin.

When all products were considered together, it was evident that over 50% of all samples were below labeled claim in one or more vitamins. Wide fluctuations in potency occurred within the same lots and the content of different vitamins in samples of the same lot had a tendency to show similar trends. There was also a tendency for samples of more recent manufacture, i. e., samples toward the right of each group, to have higher potency than the others. The significance of these points is discussed in more detail below since all samples were not analyzed at the same time.

Effect of Age.—While the data in Fig. 1 have been plotted in order of lot number, the true effect of age of product when analyzed may be obscured by other factors which also influence the potency of these products. Furthermore, samples of the same lot were not always analyzed at the same time and to segregate the effect of age, data on four products have been selected and plotted against age in Fig. 2, 3, 4, and 5. The age in months was calculated from the date of manufacture to the date of analysis for capsule C, tablets E and F, and liquid I. Since the samples were not all from the same lot nor from the same retail pharmacist, the curves may be influenced by these factors also to a certain extent.

As shown in these figures there was a tendency for certain vitamins to fall below labeled potency over a period of time. The significant point is that it occurred quite early in shelf life. Calcium *d*-pantothenate in capsule C (Fig. 2) dropped rapidly below labeled potency and by fourteen months was only 40% of its original value. The values obtained for the other vitamins fluctuated about the labeled claim but maintained a satisfactory potency for twenty-seven months. Tablet E in Fig. 3 showed fair stability for vitamin A, but a tendency to fall below labeled potency with age. Vitamin B_{12}, however, was quite unstable in this preparation, and was only 25% of labeled claim at one year. The content of other vitamins varied within the error of their estimation over the twelve-month period. Tablet F in Fig. 4 exhibited a serious drop in potency of vitamins A, C, and pyridoxine in eight months. In liquid I (Fig. 5), vitamin C, riboflavin, and niacinamide were stable and uniform over an extended period of time while vitamin A, thiamine, and vitamin B_{12} exhibited extremely poor

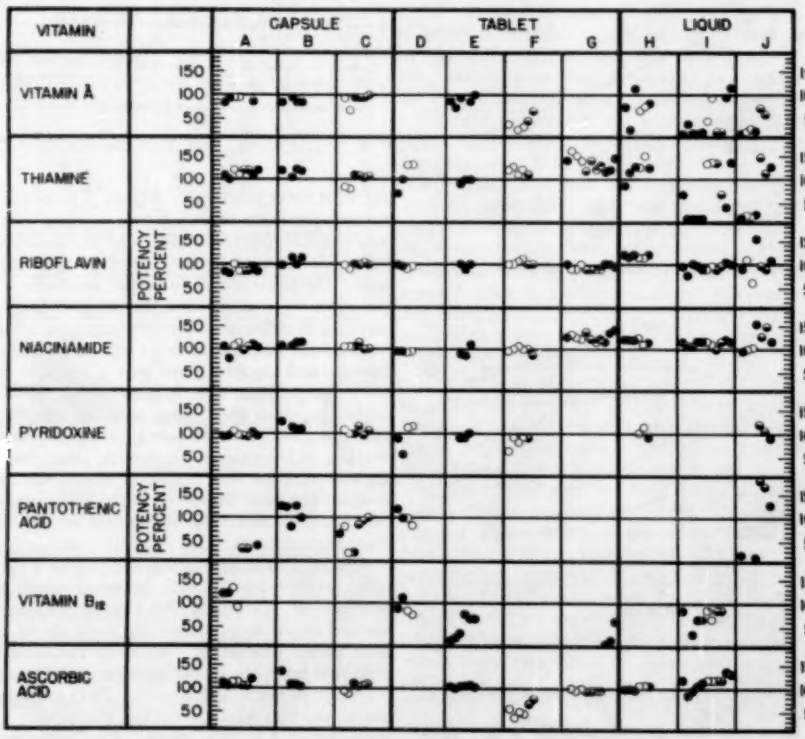

Fig. 1.—Potency of multivitamin products expressed as per cent of labeled claim. (Open dots and half open dots represent same lot number, closed dots represent different lot numbers.)

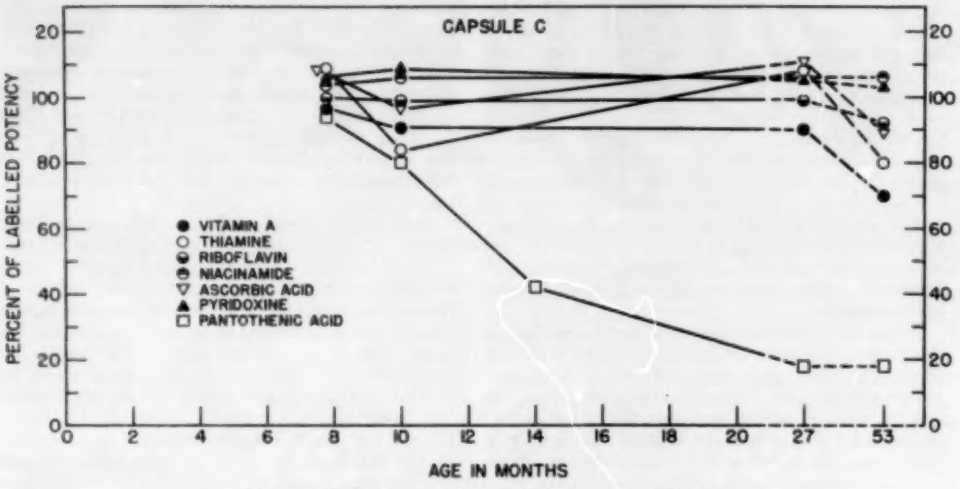

Fig. 2.—Variation in potency of Capsule C with age.

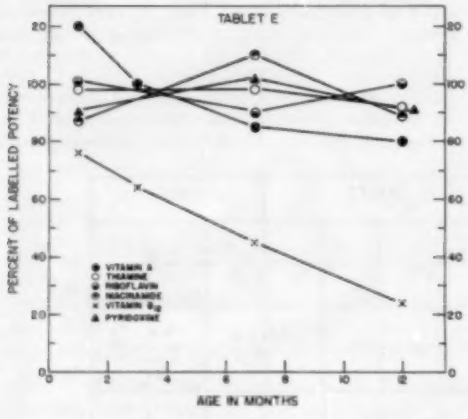

Fig. 3.—Variation in potency of Tablet E with age.

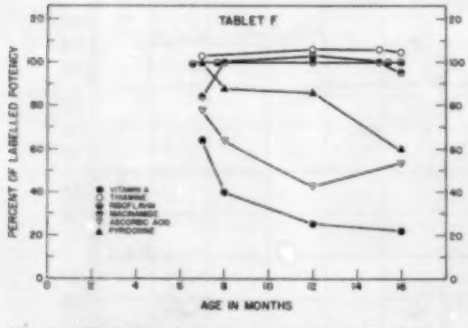

Fig. 4.—Variation in potency of Tablet F with age.

stability as shown by the marked loss in potency at the end of one year.

Effect of Store.—It was mentioned above that there appeared to be marked differences in the vitamin content of different samples of the same

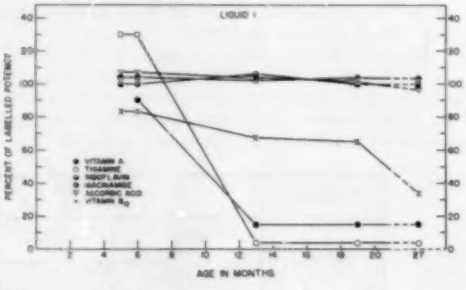

Fig. 5.—Variation in potency of Liquid I with age.

lot of several products. Figure 6 presents data on several products of which two samples of the same lot were obtained from different retail outlets. The stores designated for one product were not necessarily the same stores for other products. Capsule X, containing only vitamins A and D, showed a significant difference between samples of the same original lot held in different stores. In the case of tablets and liquids there was a marked tendency for a sample from one store to be consistently lower or higher than that from another one in vitamin content. The differences seemed particularly marked in the case of vitamin A, thiamine, vitamin C, and vitamin B_{12}. Niacin, riboflavin, and pyridoxine showed little, if any, effect presumably because they were less influenced by differences in storage conditions.

Age of Products.—Since there was a very definite relationship found between shelf life and potency of certain multivitamin preparations, it seemed important to know the shelf life of the products under discussion. Date of manufacture was obtained from the manufacturers and the age of the products is plotted in Fig. 7. Data on a few additional samples which were not completely analyzed were included in this figure. At the bottom of the figure the data have been summarized in a frequency graph. Of the 84 samples plotted, 54 were

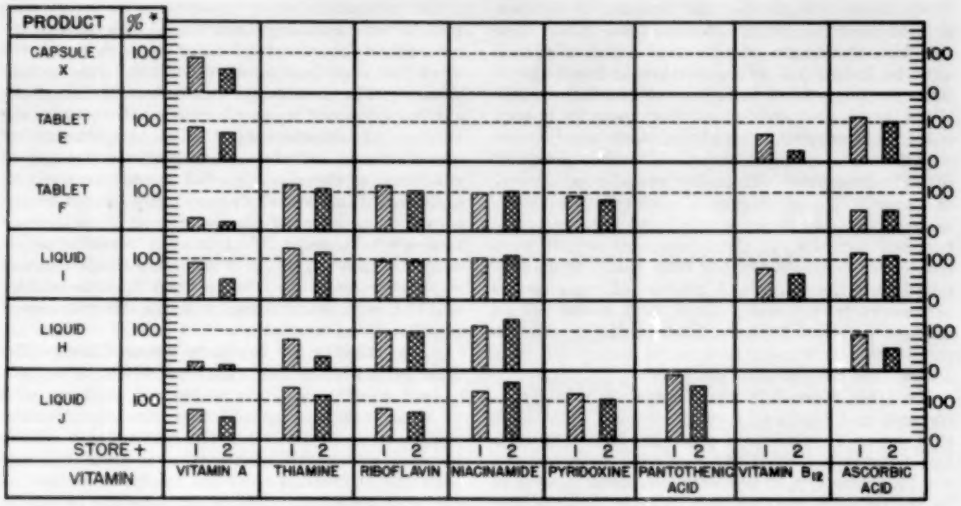

Fig. 6.—The potency of samples of the same lot purchased in different stores. (*Potency expressed as per cent of labeled claim. † The stores were not the same for each product.)

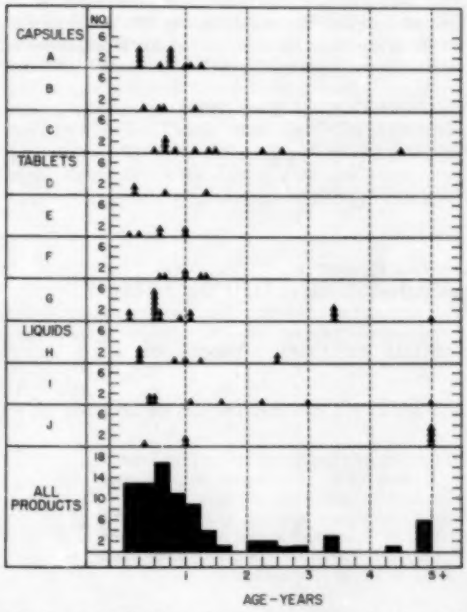

Fig. 7.—Distribution of age of products. Each point represents a single sample.

one year old or less, 14 were between one and two years old, and 16 were from two to five years old. On a percentage basis 36% were over one year old. The distributions for tablets, capsules, and liquids appear on the whole to be similar.

DISCUSSION

In discussing the significance of the results of this investigation the importance of factors affecting these data should be kept in mind. Variation in the observed results (Fig. 1) could be caused by any or all of the following factors: (1) shelf age, (2) storage conditions, (3) variation between lots of a product, and (4) experimental error of the method of assay.

Considering the data as a whole, certain observations may be made which, obviously, should apply only to the products under discussion. The potency of certain vitamins in some pharmaceuticals is markedly affected by shelf age. There are definite losses in potency in several products as early as eight months after manufacture. There are significant differences in the potency of samples of the same lot which have been purchased from different retail outlets. As would be expected, the more stable vitamins are less affected by storage time, storage conditions, and associated substances than are the less stable ones. Vitamins A and B_{12} seem to be particularly unstable in the liquids. This has been substantiated more recently with several other liquid preparations. It also confirms the work of Blitz, *et al.* (7). With the exception of pantothenic acid, vitamins in capsules are relatively stable in all factors.

The method of manufacture and the composition of individual products may also have an important bearing on stability. For example, thiamine in one liquid suffered almost complete destruction while in two tablets and one capsule it was quite stable. The loss of thiamine was traced to a relatively high concentration of sodium sulfite. In the case of another liquid, iodine appeared to cause poor stability while potassium iodide did not. In two preparations (capsule A and tablet G) containing folic acid, there appeared to be a tendency for riboflavin to be very slightly but uniformly below labeled claim. Whether there is an interaction between these two vitamins as has been suggested by Scheindlin, *et al.* (19), has not been determined. Tablet F appears to be the only product exhibiting destruction of pyridoxine or ascorbic acid. The reason for this is not clear since these vitamins are usually relatively stable in tablets.

No direct indication of the precision of the procedures used can be gained from these data. The variation in vitamin content over a period of time may be influenced by production and storage as well as analytical variation. Nevertheless, the variation was not excessive. For example, in capsule C, riboflavin, pyridoxine, and niacinamide varied over a range of about 4% over a period of fifty-three months. Thiamine varied over a range of about 20% and vitamin C about 10% over the same period. In tablet F and liquid I the variation between samples in those vitamins which were stable was considerably less than this. It may be noted that the vitamin A content of the capsules appears to be uniformly about 10% below labeled claim. Variation between laboratories in this determination is known to exist at times and may account for the situation observed.

Need for More Effective Control.—Certain conclusions may be drawn from these data. The facts that many multivitamin products have begun to lose potency less than one year after manufacture, and that over 30% of products purchased at random on the retail market are past this age indicate that a large proportion of market samples must be below labeled claim in one or more vitamins. In fact, over 50% of the samples studied were deficient in one or more vitamins. What is equally important, the data indicate that existing control facilities are not as effective as they should be in the pharmaceutical industry.

The companies whose products were included in this survey are among those known to have analytical control laboratories. In a previous paper the need for analytical control facilities was demonstrated. The results presented here demonstrate the need for still more effective control, including control of merchandising. The importance of manufacturers knowing more about the potency of products on the retail market cannot be overemphasized. This knowledge evidently cannot be obtained by accelerated storage tests or by analysis of samples held under ideal storage conditions. It can be obtained only by determining the potency of samples taken from retail shelves. Such samples will also furnish information on the effectiveness of merchandising methods.

Responsibility for Products below Claim.—The question has often been raised as to who is responsibile for maintaining the potency of products such as these. It is suggested that the manufacturer should be responsible for the maintenance of the potency of vitamin products over their "normal shelf life." Normal shelf life might be defined as the time lapse from date of manufacture to sale to the final consumer of the large bulk (possibly 95%) of stock. This time should be determined by the manufacturer from a critical survey of retail outlets for each product and not from estimated or expected figures. It would then be the responsibility of the pharmacist to ensure that his stock does not exceed its normal shelf life before it is sold. This may

TABLE I.—NUMBERS OF LOTS AND SAMPLES PROCURED IN SURVEY

	Capsules			Tablets				Liquids			
Products	A	B	C	D	E	F	G	H	I	J	Totals
Lots	6	5	4	3	6	2	7	5	9	5	52
Samples	8	5	7	4	6	6	11	6	11	7	71

TABLE II.—COMPOSITION OF PRODUCTS
(x INDICATES PRESENCE OF CONSTITUENT)

	Capsules			Tablets				Liquids		
	A[a]	B[b]	C	D	E	F	G[c]	H	I	J
Vitamin A	x	x	x	...	x	x	...	x	x	x
Vitamin D	x	x	x	...	x	...	x	x	x	x
Thiamine	x	x	x	x	x	x	x	x	x	x
Riboflavin	x	x	x	x	x	x	x	x	x	x
Niacinamide[d]	x	x	x	x	x	x	x	x	x	x
Pyridoxine	x	x	x	x	x	x	...	x	...	x
Calcium *d*-pantothenate	x	x	x	x	...	...	...	...	...	x
Vitamin B_{12}	x	...	...	x	x	...	x	...	x	...
Ascorbic acid	x	x	x	...	x	x	x	x	x	...
Folic acid	x	...	...	...	...	...	x	...	...	...
Mixed tocopherols	x	...	...	...	...	...	...	...	...	...
Liver concentrate (1:125)	...	x	...	...	...	...	...	...	...	...
Liver concentrate (1:20)	...	x	...	...	...	...	...	...	...	...
Liver fraction 2, N. F.	...	...	...	...	...	...	x	...	...	...
Calcium phosphate	x	...	...	...	...	...	...	...	...	...
Copper sulfate	x	...	...	...	...	...	x	...	...	...
Dicalcium phosphate	...	...	x	...	...	...	...	...	...	...
Ferrous sulfate	x	x	x	...	...	...	x	...	...	...
Iron phosphate	...	...	...	...	...	...	...	...	...	x
Potassium iodide	...	...	...	...	...	x	...	...	...	x[e]
Sodium iodide	x	...	...	...	x	...	...	...	...	...
Phenobarbital	...	...	...	x	...	...	...	...	...	...
Propylthiouracil	...	...	...	...	...	x	...	...	...	...

[a] Lots contained either calcium *d*-pantothenate with mixed tocopherols or folic acid with vitamin B_{12}.
[b] Vitamins A and D were contained in a separate capsule.
[c] Vitamin B_{12} and folic acid present in some lots.
[d] Liquid J contained niacin.
[e] A few samples contained iodine.

also require the cooperation and assistance of the manufacturer. The data presented here would seem to indicate a need for closer collaboration and greater interest on the part of both the manufacturer and the retailer in ensuring that products do not remain on wholesale or retail shelves for periods varying up to five years or more.

It might be questioned, under the present system of the use of lot numbers, if it will ever be possible or practical to ensure that products which pass their normal shelf life will be returned consistently to the manufacturer. The pharmacist would have no easy way of identifying which products he had just received or which he had had for a year or more. It appears obvious that vitamin products will always suffer from instability to a greater or less degree and changes of formulation or the use of higher overages would never completely eliminate the possibility that a customer might by chance purchase a product that had passed its shelf life and that would be below label claim. It is suggested that an expiration date, conspicuously displayed on the label, would do much to ensure that the consumer obtains a product which meets label claim. This has already been suggested by Bagnall and Stock (8) in Great Britain for halibut liver oil capsules. An expiration date, based on the stability of the drug, is required in Canada at present for all antibiotics (20). Morrell (21) has pointed out possible advantages of its wider application to all products suffering from instability. It would seem particularly applicable to multivitamin preparations where incompatibility is superimposed on the inherent instability of individual vitamins. An expiration date would offer a simple means of checking the age of products for both the retailer and consumer. Furthermore, the expiration date would indicate directly where the responsibility lay for any products found below label claim. This should be mutually satisfactory to the conscientious manufacturer and pharmacist. The fact that at least two large manufacturers in Canada have just recently begun using an expiration date on liquid multivitamin preparations of the type discussed here indicates that the idea is practical and warrants the serious consideration of all manufacturers of multivitamin products.

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Investigation of Drug Release from Solids*

I. Some Factors Influencing the Dissolution Rate

By EUGENE L. PARROTT,† DALE E. WURSTER, and T. HIGUCHI

A method for determining the dissolution rate and the underlying physical-chemical theories are presented. The velocity constant for the conditons employed in the procedure and the dissolution rate of compressed benzoic acid tablets were determined. In the density range investigated the dissolution rate was essentially independent of the tablet density. When neutral ionic and nonionic organic compounds were employed as additives the dissolution rate was linearly dependent upon the solubility of benzoic acid in the particular solvent system.

AN ATTEMPT has been made in this study to investigate, *in vitro*, some of the factors affecting the dissolution rate of slightly-soluble acidic drugs. In this initial report the method of investigation together with its underlying physical-chemical theories are presented.

Most pharmaceutical studies of the release of a drug from a solid system have dealt mainly with the time required for disintegration of a tablet. Elaborate studies have been conducted both in apparatus constructed to simulate the human digestive tract and in artificial media (1, 2). Some workers (3) have concluded that artificial gastric juice did not produce sufficiently consistent ef-

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fects to warrant its use in place of distilled water. Hoehn (4) suggested that aging changes the disintegration time. Sperandio (5) stated that the efficiency of a tablet is to a great extent influenced by the speed with which it disintegrates, and he made the important distinction between disintegration, the mere breaking up into small particles or granulations, and solution.

In treating drug absorption as a free diffusional process, Edwards (6) calculated that the drug concentration on both sides of the epithelial layer of the intestines approached equilibrium in a remarkably short time. He, therefore, contended that a drug is absorbed almost as rapidly as it dissolves. Thus, if it is assumed that therapeutic response immediately follows drug absorption, the governing rate largely responsible for controlling the response is the rate of dissolution.

While the disintegration time of a tablet does influence the rate of drug release to the body, a most important aspect is the rate of release from the primary drug particle since solution of the drug is essential in order for absorption to take place. Furthermore, this aspect must be considered whenever solid drug particles are introduced into the body irrespective of whether the drug is administered orally, by injection or by some other means. Thus, the release of a drug from the primary particle and its subsequent availability to the body is governed by the dissolution rate of the particle.

It would be most useful from both the standpoint of preparation and administration of drugs to know more concerning the factors which affect the dissolution rate of drug particles. Such factors would include the characteristics of both the particle and the dissolving medium.

METHOD OF STUDY

To study the dissolution rate of a substance in various solutions a simple yet reasonably accurate method to determine the rate was necessary. The general procedure which was finally developed and employed for this purpose consisted of placing the test substance in the form of a compressed tablet in an agitated aqueous medium which was kept at constant temperature. The solvent phase was frequently renewed to prevent an appreciable build-up of the solute concentration. Measurements of the weight and radius, in the case of spherical tablets, were taken initially and at various time intervals. An optic micrometer was used to measure the radius. The extent of dissolution was further checked by titration of the solvent phase. There was no significant variation between the titration and direct weighing methods.

No prior study appears to have been made with the primary objective of determining the dissolution behavior of an isotropic crystalline mass. Nearly all of the work carried out in this area has been devoted to rates of solution of isolated crystals or of a single crystal.

In the case where the amount of solute dissolved is small enough to be considered negligible so that the solute concentration may be treated as constant, Hixson and Crowell (7) have derived the following relationship for this special case of the Noyes-Whitney law (8). It was assumed that solution takes place normal to the surface of a particle and, therefore, a constant shape is maintained throughout the dissolution process. The rate is then proportional to the surface of the particle. Thus,

$$-\frac{dw}{dt} = 3KS = 3KaW^{2/3} \qquad (1)$$

where $3K$ represents the dissolution rate per unit of surface (Gm./hr./cm.^{2}), S is the surface at time, t, W is the weight at time, t, and a is the constant

$$\frac{asv}{\rho^{2/3}} = \frac{4.85}{\rho^{2/3}}$$

On integration between the limits W_0 and W

$$Kat = W_0^{1/3} - W^{1/3} \qquad (2)$$

Here W_0 and W are the initial weight at time $t = 0$ and the weight at time, t, respectively, and K is the velocity constant for a given set of conditions.

If it is assumed that the compressed, somewhat porous, tablet behaved essentially like compact homogeneous solids to which the above equations apply, the rate of solution of the compressed solids can be determined by use of Eq. 1. Such an assumption would be justified if the pore openings of the porous mass were always filled with solvent saturated with respect to the solid. Evidence supporting this belief is presented later in this paper.

RESULTS AND DISCUSSION

By use of the procedure outlined above studies were carried out on the dissolution behavior of compressed benzoic acid spheres to determine whether the results obtained by the method conformed to the theory. The influence of the particle density and some neutral additives on the rate of solution were then investigated.

Shape Maintenance During Dissolution.—In the derivation of Eq. 1 it is tacitly assumed that the geometric shape of the dissolving particle does not significantly change during the dissolution process. That such is the case appears to be supported by experimental evidence.

From a visual inspection of a spherical benzoic acid tablet during its dissolution in water it was evident that the particle retained an essentially constant spherical shape. This is further supported by the fact that when the volume of the tablet was calculated from the measurement of its radius and plotted against its weight during dissolution as shown in Fig. 1, a straight line passing through the origin was obtained. The tablet used for this particular graph was examined until 93% of its original weight dissolved.

A constant shape-volume relationship is not unique to either a spherical shape or a specific chemical such as benzoic acid since similar results

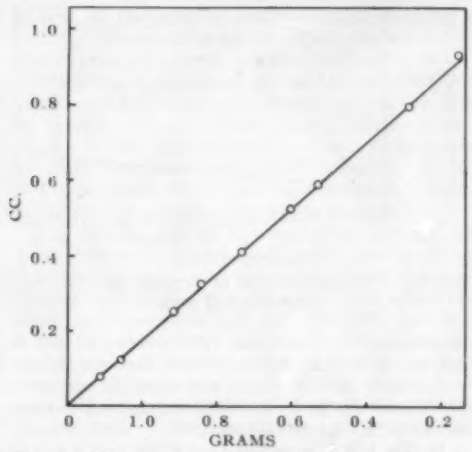

Fig. 1.—Volume vs. weight of spherical benzoic acid tablet during dissolution.

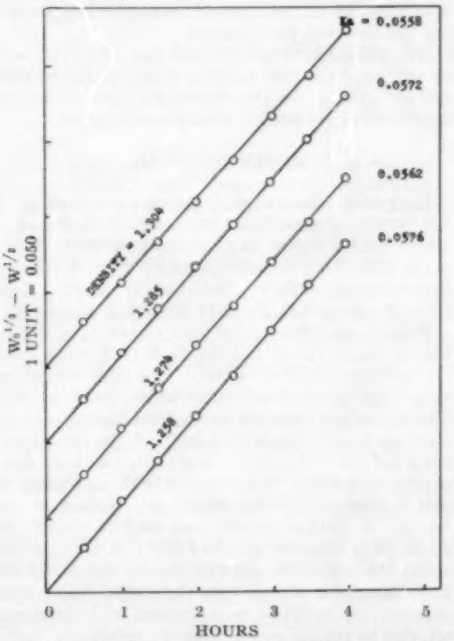

Fig. 2.—Evaluation of Ka from the slope of $W_0^{1/3} - W^{1/3}$ vs. time. Each curve intersects the sliding scale on the ordinate at zero.

were obtained with cylindrical tablets of aspirin and salicylic acid.

Velocity Constant.—Under a given set of experimental conditions the velocity constant, K, of the Hixon-Crowell Eq. 2 can be readily evaluated from a plot of $W_0^{1/3} - W^{1/3}$ against time. This was done for sixteen different dissolution runs on spherical benzoic acid tablets as shown, for example, in Fig. 2. The calculated K values for all of the determinations are shown in Table I, the average value being 0.0142. Since spherical tablets were used

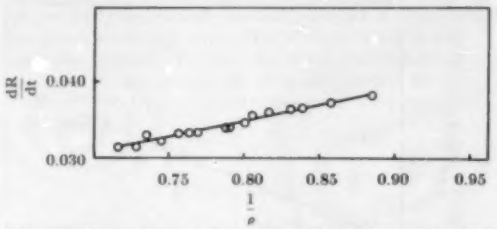

Fig. 3.—Rate of change of radius of a spherical tablet with time vs. the reciprocal of its density.

TABLE I.—DETERMINATION OF K BY WEIGHT METHOD

Tablet No.	$\rho_{3/3}$	Ka	$\frac{4.85}{\rho^{1/3}}$	K
485	1.194	0.0558	4.06	0.0137
459	1.182	0.0572	4.10	0.0139
314	1.125	0.0608	4.31	0.0141
428	1.175	0.0562	4.13	0.0135
468	1.165	0.0576	4.16	0.0139
445	1.164	0.0605	4.16	0.0145
429	1.154	0.0608	4.20	0.0145
420	1.149	0.0610	4.22	0.0145
422	1.141	0.0625	4.25	0.0147
399	1.114	0.0622	4.35	0.0143
334	1.101	0.0672	4.41	0.0152
338	1.110	0.0650	4.35	0.0149
344	1.083	0.0662	4.48	0.0147
536	1.081	0.0627	4.485	0.0140
530	1.067	0.0632	4.54	0.0139
532	1.045	0.0630	4.64	0.0136

$$a_{sv} = 4.85 = \pi \left[\frac{1}{(\pi/6)}\right]^{1/3}$$

The same constant can also be determined from the rate of decrease of the radius of the tablet by use of the equation

$$\frac{dR}{dt} = \frac{3K}{\rho} \qquad (3)$$

The average value of the velocity constant, K, by this method was found to be 0.0137.

The rate of dissolution in this procedure may be expressed as

$$\frac{dw}{dt} = 3KS = 0.0424S$$

Therefore, the dissolution rate, $3K$, for benzoic acid in water under these conditions was

$$0.0424 \text{ Gm./hr./cm. and } K = \frac{0.0424}{3} = 0.0141$$

Influence of Tablet Density on Dissolution Rate.—As expected from Eq. 3 a plot of $1/\rho$ against dR/dt should give a linear relationship. This relationship was confirmed experimentally as shown in Fig. 3. The tablets of differing densities (1.054–1.304) were prepared by compressing the benzoic acid with different forces. Over the density range available with tablets prepared by compression, the dissolution rate was essentially independent of density. Thus, although with less dense tablets a greater increment of volume was dissolved per unit of time than with the more dense tablets, an

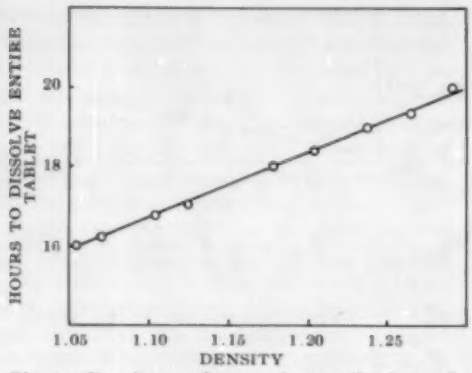

Fig. 4.—Density vs. time required to dissolve entire tablet.

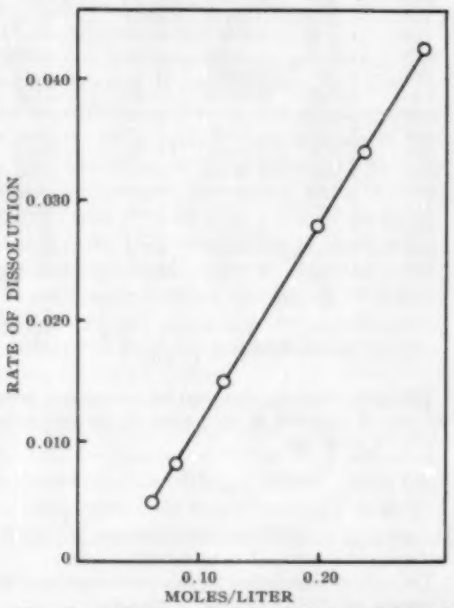

Fig. 5.—Influence of the solubility of benzoic acid on the dissolution rate in sodium sulfate solutions.

equal weight of material was dissolved per unit time from a given surface area for both cases.

The time required to dissolve the whole tablet was directly proportional to its weight, and since all tablets initially were of the same volume, the product of the weight and a constant was equivalent to the density. In other words, the total time required to dissolve a tablet varied directly as the density as shown in Fig. 4.

Effect of Neutral Ionic and Nonionic Organic Compounds.—The dissolution rate of benzoic acid was decreased when various concentrations of sodium chloride, sodium sulfate, and dextrose were added to the dissolving medium. It was observed that sodium sulfate was more effective than dextrose and sodium chloride was the least effective in

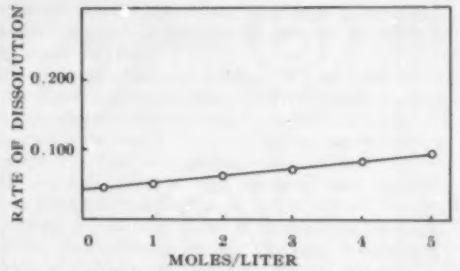

Fig. 6.—Dissolution rate of benzoic acid vs. concentration of urea.

decreasing the dissolution rate of benzoic acid in water. From Fig. 5 it is evident that the dissolution rate is linearly dependent upon the solubility of benzoic acid in the particular dissolving medium as would be expected theoretically.

In Fig. 6 it is observed that when urea was employed as an additive an increase in the dissolution rate resulted. Because of the small dissociation constant of urea it would not seem that the slight alkalinity of the compound was alone responsible for the observed rate increase.

The influence of reactive alkaline additives, both in the presence and absence of an excess of electrolyte (NaCl), on the dissolution rate of benzoic acid will be presented in a subsequent paper.

EXPERIMENTAL

Reagents.—Benzoic acid, primary standard, m. p. 122°; recrystallized salicylic acid, U. S. P., m. p. 159°; recrystallized acetylsalicylic acid, U. S. P., m. p. 133–135°; sodium chloride, U. S. P.; anhydrous sodium sulfate; dextrose, U. S. P.; urea, U. S. P., m. p. 132.5°; 0.01 *N* sodium hydroxide.

Procedure.—The tablet was placed in a 2-liter, three-necked, round bottom flask containing 2,000 ml. of boiled distilled water. This flask was maintained in a constant temperature bath at 25°. The liquid was agitated by a stirrer having a 1/4-in. steel shaft and two blades, each of the dimensions 1.0 x 1.5 cm. The stirrer was coated with an inert plastic to prevent any reaction with the solutions and immersed 5.3 cm. below the surface of the liquid. A Labline motor was used to rotate the stirrer at a constant speed of 550 r. p. m. At this speed the tablet did not rest on the bottom of the flask but rotated freely in the liquid. The temperature of the liquid was checked and the stirrer allowed to rotate several minutes before the tablet was placed in the solvent.

At the end of a given time the tablet was withdrawn from the solution, thoroughly dried by blotting with a soft absorbent cloth and placed under an optic micrometer (Gaertner Scientific Corp.) on a specially constructed substage which permitted rotation in a single plane without disturbing the tablet. The tablet was measured in a total of sixteen positions in two planes to 0.0001 cm. The average of these readings expressed to 0.001 cm. was used as the diameter of the sphere.

The tablet was then weighed and returned to a fresh 2,000 ml. of solution to begin another cycle.

To determine if the weight dissolved was the same as the difference between the initial weight and the

weight of the tablet at a given time, a 25-ml. aliquot of the solution was titrated with 0.01 *N* NaOH. There was no significant difference between the titration and direct weighing methods.

When the influence of sodium chloride, sodium sulfate, dextrose, and urea on the dissolution rate of benzoic acid was investigated, solutions containing various concentrations of these agents were accurately prepared using boiled distilled water. These solutions were then substituted for water as the solvent system in the above procedure.

The tablets used in this work were compressed with a Stokes (Model A-3) single punch tablet machine equipped with special half-inch spherical punches adjusted so that the rim formed where the upper and lower punches did not touch was at a minimum. The tablet machine was instrumented, as previously reported by Higuchi, *et al.* (9), so that oscillograph recordings of the compressional force were obtained. Because of the bulkiness of the benzoic acid powder the tablets were prepared by "slugging"; the "slugs" were then ground and those particles which passed through a No. 20 standard sieve but not a No. 40 were used to prepare the tablets. In the case where the dissolution rate of cylindrical salicylic acid and aspirin tablets was studied, $^3/_8$-in. flat punches were used to form the tablets.

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Mannich Bases from 5-Nitro-2-Acetylfuran and 5-Nitro-2-Furfuralacetone*

By H. CECIL CALDWELL† and W. LEWIS NOBLES

Eleven Mannich bases derived from 5-nitro-2-acetylfuran and 5-nitro-2-furfuralacetone have been prepared using various dialkyl and heterocyclic secondary amines. These β-aminoketones are to be screened for possible pharmacodynamic activity.

RECENT STUDIES (1–4) have indicated that certain β-amino ketones and their derivatives possess both chemotherapeutic and pharmacodynamic activity. Such compounds may be prepared rapidly by means of the Mannich reaction, which may be indicated as follows:

$$RCOCH_3 + HCHO + R_2'NH\cdot HCl \longrightarrow RCOCH_2CH_2NR_2'\cdot HCl + H_2O$$

The rather extensive literature dealing with this reaction has been reviewed by Blicke (5).

Recently, Denton and his associates (4) published a series of papers dealing with the preparation and pharmacological evaluation of various β-dialkylaminopropiophenones and their derivatives. Many of these proved to be very effective antispasmodic agents. Mannich and Lammering (1) had found much earlier that β-piperidylpropiophenone hydrochloride, a substance easily obtained from acetophenone, piperidine hydrochloride, and paraformaldehyde, possessed local anesthetic activity. Substituting 2-acetylpyrrole for acetophenone in the above, Blicke and Blake (2) obtained a substance with marked local anesthetic properties. Levvy and Nisbet (3) prepared a series of Mannich bases from 2-acetylthiophene and 2-acetylfuran and ascertained that several of their compounds demonstrated local anesthetic activity.

Since many derivatives of furan have recently become available in experimental quantities, considerable interest has been evidenced in the preparation of furan analogs of compounds of the benzene series which possess medicinal value.

In addition to the relationship of the Mannich bases described in this paper to the pharmacodynamic agents already mentioned, these compounds, like nitrofurazone, are derivatives of 5-nitrofuran.[1]

In consideration of these facts the first portion of this work was designed to prepare the nitrofuran analogs (I) of certain β-dialkylaminopropiophenones:

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Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
This work was supported in part by a grant from the Cyrus M. Warren Fund of the American Academy of Arts and Sciences.
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[1] Since submission of this manuscript, a reference, *Chem. Abstr.*, **48**, 12809(1954)) indicates that U. S. pat. 2,663,710 has been assigned to cover two of the Mannich bases mentioned in this paper, i. e., the dimethylamine and the piperidine Mannich bases derived from 5-nitro-2-acetylfuran. The semicarbazones of these Mannich bases are regarded as being chemotherapeutically active water-soluble nitrofuran compounds which are suitable for parenteral use.

TABLE I.—5-NITRO-2-(β-DIALKYLAMINOPROPIONYL) FURAN HYDROCHLORIDES[a]

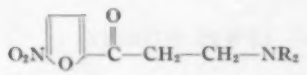
No.	Amino Group	m. p.,[b] °C.	Yield, %	Formula	Analyses[c] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	Dimethylamino	195–197	20	$C_9H_{13}N_2O_4$·HCl	43.46	43.58	5.27	5.19
2	Diisopropylamino[d]	192	63	$C_{13}H_{20}N_2O_4$·HCl	51.23	51.16	6.95	6.87
3	Dibutylamino[d]	177–179	25	$C_{15}H_{24}N_2O_4$·HCl	54.13	54.42	7.57	7.71
4	4-Morpholinyl	194	51	$C_{11}H_{14}N_2O_5$·HCl	45.44	45.67	5.20	5.12
5	1-Piperidyl	202–203	50	$C_{12}H_{16}N_2O_4$·HCl	49.91	49.76	5.94	5.43
6	2-Methylpiperidyl	196	35	$C_{13}H_{18}N_2O_4$·HCl	51.57	51.85	6.33	6.52

[a] The intermediate ketone, 5-nitro-2-acetylfuran, was supplied through the courtesy of Eaton Laboratories.
[b] All melting points were taken on a Fisher-Johns apparatus and are uncorrected.
[c] Analyses by Weiler and Strauss, Oxford, England.
[d] Amine supplied through the courtesy of Carbide and Carbon Chemicals.

TABLE II.—5-DIALKYLAMINO-1-(5-NITRO-2-FURYL)-1-PENTEN-3-ONE HYDROCHLORIDES[a]

No.	Amino Group	m. p.,[b] °C.	Yield, %	Formula	Analyses[c] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
7	Dimethylamino	198–200	20	$C_{11}H_{14}N_2O_4$.HCl	48.09	47.87	5.50	5.29
8	Diisopropylamino[d]	181	20	$C_{15}H_{22}N_2O_4$.HCl	54.45	54.62	7.01	7.12
9	4-Morpholinyl	196–198	58	$C_{13}H_{16}N_2O_5$.HCl	49.29	49.86	5.41	5.29
10	1-Piperidyl	205–207	53	$C_{14}H_{18}N_2O_4$.HCl	53.42	53.65	6.08	5.99
11	2-Methylpiperidyl	210–211	44	$C_{15}H_{20}N_2O_4$.HCl	54.79	54.68	6.44	6.36

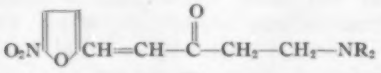
[a] The intermediate ketone, 5-nitro-2-furfuralacetone, was obtained through the courtesy of Eaton Laboratories.
[b] All melting points were taken on a Fisher-Johns apparatus and are uncorrected.
[c] Analyses by Weiler and Strauss, Oxford, England.
[d] Amine supplied through the courtesy of Carbide and Carbon Chemicals.



I

These compounds are listed in Table I.

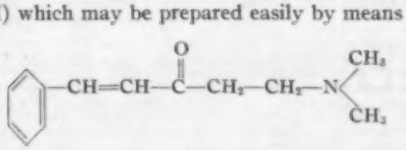
The second portion of this work dealt with the preparation of vinylogs (II) of the above com-



II

pounds. This group of compounds was prepared in an effort to determine to what extent the principle of vinylogy, as advanced by Fuson (6) in regard to chemical reactivity, could be extended in this specific area of pharmacological activity. Some very interesting results have been reported in studies of vinylogous pharmacodynamic substances by others (7).

Schraufstatter and Deutsch (8) demonstrated that certain α,β-unsaturated ketones possessed definite antibacterial activity. Furthermore, interest in this type of compound was increased as a result of the *in vitro* antibacterial activity recently reported by Burckhalter and Johnson (9) for 5-dimethylamino-1-phenyl-1-penten-2-one (III) which may be prepared easily by means of



III

the Mannich reaction. These vinylogs are set forth in Table II.

DISCUSSION

These 11 related β-amino ketones were all prepared by means of the Mannich reaction, using paraformaldehyde, the desired secondary amine, and either 5-nitro-2-acetylfuran or 5-nitro-2-furfuralacetone.

The procedure employed was very similar to that used by Levvy and Nisbet (3) in preparing Mannich bases from 2-acetylfuran. The yield of the desired compound was markedly influenced by both the manner of addition of the paraformaldehyde and the length of time allowed for refluxing. When Maxwell's procedure (10), which involves the addition of the paraformaldehyde at once and two hours of refluxing, was employed, little or none of the desired products was obtained. This observation supports the belief of Lieberman and Wagner (11) that the success of the Mannich reaction depends on the maintenance of a proper acid-base relationship. They point out that the Mannich reaction probably involves a dual catalysis in an amphoteric system. This is, of course, possible during this reaction, for one of the reactants is basic and the other acidic, at least to a degree. Lieberman and Wagner (11) point out that a knowledge of the acid-base relationship is essential to the understanding of the nature of this reaction. These workers indicate that excess acidity or basicity will check, impede, or prevent the Mannich reaction. If unfavorable acid-base relationships prevail, it has been demonstrated that the yield of expected product will be considerably diminished. The decreased yield of the desired product may well be a result of an unfavorable acid-base relationship which slows down or prevents the condensation

of aldehyde and amine. Perhaps, as Lieberman and Wagner suggest (11), the introduction of formaldehyde (or paraformaldehyde) during the reaction not only restores the concentration of a rapidly depleting primary reactant but also favorably affects an otherwise deteriorating acid-base relationship.

EXPERIMENTAL

The Mannich bases were all prepared by one or the other of the following two procedures. Basically, as will be evident from an inspection of the actual procedure, these methods differ only in regard to the manner in which the amine is placed in the reaction vessel.

Procedure A.—The amino ketones were prepared by the general procedure of refluxing 0.1 mole of the ketone with 0.1 mole of the amine hydrochloride and 1.5 Gm. of paraformaldehyde in 20–25 ml. of absolute alcohol containing one or two drops of concentrated hydrochloric acid for fifteen minutes. Another 1.5-Gm. portion of paraformaldehyde was added and refluxing continued for an additional thirty minutes. At the end of this time, the warm solution was poured into 100 ml. of acetone and the contents of the flask were cooled to room temperature. The mixture was then refrigerated until the solid product precipitated. The solid thus obtained was removed by filtration and recrystallized to analytical purity from an alcohol-acetone solution.

Procedure B.—This method was utilized only when amines, not generally available as the crystalline hydrochlorides, were utilized. The secondary amine was dissolved in 20–25 ml. of alcohol, and this solution was made slightly acidic to litmus by the dropwise addition of concentrated hydrochloric acid. To this alcoholic solution was added the ketone and paraformaldehyde and the general procedure of A was utilized from this point on.

SUMMARY

Eleven Mannich bases derived from 5-nitro-2-acetylfuran and 5-nitrofurfuralacetone, and using the desired secondary amines, have been prepared.

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The Stability of Neomycin in Several Pharmaceutical Preparations*

By ROSE MARY SIMONE and ROBERT P. POPINO

Neomycin is a water-soluble polybasic antibiotic relatively stable in solution over a range of *p*H 2.0 to 9.0. It is most active in alkaline solution and is active against Gram-positive and Gram-negative bacteria, but is inactive against fungi. In ointment bases, i. e., white petrolatum, polyethylene glycol, anhydrous lanolin, and Jelenes, neomycin was found to be stable for periods approximating two years at room temperature. Neomycin oil suspensions were found to be stable for periods of approximately one year at room temperature. Neomycin tablets and troches containing the usual excipients were stable for periods of two years at room temperature and at 56°. Aqueous nasal preparations, mouth washes, and tinctures were found to be stable for one year at room temperature.

NEOMYCIN is a water-soluble polybasic antibiotic first prepared and isolated in 1949 by Waksman and his associates (1) at the New Jersey Agricultural Experiment Station at Rutgers University. Neomycin sulfate is an off-white amorphous powder stable in the dry state for at least three years when stored at room temperature and fairly stable in solution over a *p*H range of from 2.0 to 9.0.

Although the ultimate structure of the neomycin molecule has not been elucidated, the chemical properties of the compound may be summarized as follows: Neomycin gives positive Molisch and carbazole tests for carbohydrate residues; it gives negative maltol, Sakaguchi, and Elson-Morgan tests distinguishing it from streptomycin. It gives a positive ninhydrin test and Dutcher, *et al.* (2), concluded that all of the nitrogen is present in primary amino groups.

Countercurrent distribution studies have indicated that the antibiotic isolated as neomycin represents a mixture of several biologically active entities. The substance designated as neomycin

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A by Peck, *et al.* (3), is now known to be identical with neamine, a degradation product of neomycin (Leach and Teeters (4)), and is not a member of the neomycin complex. Regna and Murphy (5) succeeded in isolating a homogeneous active substance which they designated as neomycin B. Subsequently, Dutcher, *et al.* (2), reported the preparation of two homogeneous biologically active fractions, neomycins B and C, which are apparently isomeric.

Swart, *et al.* (6), studied a number of neomycin preparations by forty-nine transfer countercurrent distribution between borate buffer and stearic acid in Pentasol[1] (synthetic amyl alcohols), using the method of alternate withdrawals in a twenty-five tube Craig machine. Neomycin A was found to be a homogeneous material showing a single peak around tube 33. Neomycin was characterized as a mixture of closely related substances peaks of which occur around tubes 36, 40, and 46.

Production lots of neomycin salts prepared in the laboratories of American Cyanamid Company and used in these stability studies have been analyzed by the partition method of Swart, *et al.* and found to be the clinically effective neomycin complex described by Swart or the neomycin B described by Regna.

Neomycin has proved to be a clinically useful drug against certain Gram-positive organisms and more so against Gram-negative organisms, particularly infections due to *Proteus vulgaris*, *Escherichia coli*, and *Aerobacter aerogenes*. The administration of neomycin orally and topically has not resulted in toxic side reactions. The incidence of allergic reactions to topically administered neomycin is very low. These factors combined with the relatively stable nature of the drug in solution over a fairly wide range of *p*H values and the fact that the drug is not inactivated by bacterial enzymes have made the use of neomycin most promising in the field of dermatologic infection. The topical administration of neomycin either as wet dressings or ointments has been recommended for the treatment of susceptible infections of the skin and eye, including pyogenic or secondarily infected stasis dermatoses, impetigo, wounds, burns, ulcers (varicose or trophic), conjunctivitis, blepharitis, and sty.

In addition, neomycin is not appreciably absorbed from the intestinal tract after oral administration, therefore its role as an intestinal disinfectant is becoming increasingly important.

Because of the relatively wide range of oral and topical applications possible with neomycin, it seemed desirable to obtain stability data for this antibiotic in a wide variety of dosage forms containing neomycin alone and also in combination with other drugs. Accordingly, extensive stability tests have been conducted for the following pharmaceutical forms containing neomycin: aqueous solutions, ointments, and oleaginous suspensions and tablets (Tables I–IX).

EXPERIMENTAL

All of the preparations described in this study were formulated in our laboratories either expressly for stability studies or in the course of the development of new products containing neomycin. Unless otherwise specified, neomycin sulfate was used in the formulation studied.

Stability samples at elevated temperatures were stored in small rooms thermostatically maintained at 37, 45, and 56°. Room temperature samples were stored in a portion of an air-conditioned room in which the temperature varied from 21 to 24°.

Samples were assayed biologically for neomycin content by the method developed in our laboratories using the cup-plate assay with *Bacillus subtilis* as the test organism (7). When neomycin was formulated in aqueous vehicles, compressed forms, or water-miscible bases capable of solution, samples were diluted or dissolved at appropriate levels and assayed directly.

When neomycin was formulated alone in water-immiscible bases, samples were first dissolved in a mixture consisting of equal parts of ether and petroleum ether and the solvent solution extracted several times with a phosphate buffer (*p*H 8.0). The phosphate buffer was then assayed directly for neomycin.

When neomycin was formulated with other antibiotics in water-immiscible bases, the ether-petroleum ether solution was extracted with distilled water. In the case of neomycin-bacitracin mixtures, the aqueous extract was diluted with *p*H 8.0 phosphate buffer for assay of the neomycin component since bacitracin had been found to be inactive against *B. subtilis* in this procedure. Conversely, the aqueous extract was diluted with *p*H 6.0 buffer for the disc-plate assay of bacitracin using *Micrococcus flavus*. It had been our experience that the presence of as much as 20 μg./ml. of neomycin did not appear to interfere with the assay of bacitracin solutions at 1 u./ml.

In the case of neomycin-penicillin mixtures, the penicillin component of the aqueous extract was inactivated with penicillinase to prevent interference with the neomycin assay. In the concentrations employed, there had been no evidence of interference of neomycin with the cup-plate assay of penicillin using *B. subtilis* as the test organism.

In the case of newly prepared neomycin-dihydrostreptomycin mixtures, the dihydrostreptomycin content of the aqueous extract was determined chemically by the Monastero (8) method and the neomycin component was determined chemically by acid hydrolysis and determination of the furfural produced (9). However, the acid hydrolysis-furfural method cannot be used for determining the neomycin content of samples in which inactivation has occurred because the inactivation products

[1] Manufactured by Sharples Chemical Company.

TABLE I.—STABILITY OF NEOMYCIN SULFATE AND NEOMYCIN HYDROCHLORIDE IN AQUEOUS SOLUTIONS

Composition of Vehicle	Neomycin Sulfate at 3.25 mg. of Base per ml. Stored 24 Months				Neomycin Hydrochloride at 3.50 mg. of Base per ml. Stored 14 Months			
	At 23°		At 45°		At 23°		At 45°	
	Activity Loss, %	Soln. Color	Activity Loss, %	Soln. Color	Activity Loss, %	Soln. Color	Activity Loss, %	Soln. Color
Distilled water	None	Water-white	27	Orange	None	Water-white	10	Amber
*p*H 4.0 buffer[a]	12	Water-white	94	Orange	None	Light yellow	90	Amber
*p*H 6.0 buffer[a]	None	Water-white	50	Yellow	None	Light yellow	28	Amber
*p*H 8.0 buffer[a]	None	Very slight yellow	88	Orange	18	Yellow	39	Orange
Sodium metabisulfite (0.1%)	17	Water-white	62	Orange	None	Water-white	12	Light Yellow
Sodium metabisulfite (0.1%) in *p*H 6.0 buffer[a]	12	Light yellow	65	Orange	None	Water-white	None	Amber
Sodium metabisulfite (0.1%), sodium citrate (3%)	None	Yellow	38	Yellow	None	Light yellow	None	Yellow

[a] MacIlvaine's buffer mixtures: disodium phosphate–citric acid.

TABLE II.—STABILITY OF NEOMYCIN SULFATE OR HYDROCHLORIDE IN AQUEOUS PHARMACEUTICAL FORMULATIONS

Dosage Form	Neomycin Base Content, mg./ml.	Other Ingredients	Neomycin Stability		
			Temperature, ° C.	Time, Mos.	Activity Loss
Nasal drops	3.3	Methyl (0.1%) and propyl (0.02%) parabens; normal saline solution	23, 45	16	None
Nasal drops	3.3	Methyl (0.1%) and propyl (0.02%) parabens; ephedrine sulfate (1.0%); normal saline soln.	23, 45	16	None
Mouth wash	3.3	Methyl (0.1%) and propyl (0.02%) parabens; cinnamon oil (0.025%); clove oil (0.05%); ethanol (5%). M. M. R. Red Color No. 2	23	19	None
Mouth wash	3.3	Methyl paraben (0.02%), zinc sulfate (0.2%)	23	19	None
Mouth wash	3.3	Methyl (0.1%) and propyl (0.02%) parabens; Cinnamon oil (0.025%); clove oil (0.05%); ethanol (5%)	23, 45	19	None
Mouth wash	3.3	Dobell's solution	23	8	Unstable
Tincture	70[a]	Ethanol (50%); acetone (10%); water (40%)	23	12	None
Tincture	70[a]	Ethanol (50%); acetone (10%); water (40%); and red or yellow dye	23	12	None
Tincture	70[a]	Ethanol (40%); acetone (10%); water (50%)	23	6	None
Tincture	70[a]	Ethanol (40%); acetone (10%); water (50%); FDC Red No. 1, Yellow No. 1, or Yellow No. 5	23	6	None
Otic	3.3	Neomycin *p*-aminosalicylate, propylene glycol	23	9	30%
Otic	3.3	Neomycin *p*-aminosalicylate, propylene glycol, methyl (0.1%) and propyl parabens (0.05%)	23	9	30%
Otic	3.3	Neomycin *p*-aminosalicylate, benzocaine (1%), propylene glycol, methyl (0.1%) and propyl parabens (0.05%)	23	8	30%

[a] Tinctures were prepared using neomycin hydrochloride.

give spuriously high neomycin results. It is recommended that the recently published method of the Food and Drug Administration (10) be used for the assay of the neomycin component of neomycin-dihydrostreptomycin mixtures. This method employs a plate assay with a dihydrostreptomycin-resistant strain of *Micrococcus pyogenes*.

In compiling the stability data, individual results within ±10% of the original neomycin potency have been considered to represent no activity loss. The limits of error of the plate assay for neomycin are ±15%.

TABLE III.—STABILITY OF NEOMYCIN IN HYDROPHILIC OINTMENT BASES

Neomycin Base Content	Composition of Ointment	Stability Data. No Loss at 23° for
3.3 mg./Gm.	Polyethylene glycol base, U. S. P.	34 mos.
3.3 mg./Gm.	Jelene® (water absorption base)[a]	15 mos.
5.0 mg./Gm.	Glyco-Jelene® base[a]	15 mos.
5.0 mg./Gm.	Carboxymethylcellulose gel (5%)[b]	14 mos.
3.3 mg./Gm.	Carboxymethylcellulose gel (6%), glycerin (6%)[b]	12 mos.
3.3 mg./Gm.	Carboxymethylcellulose gel (5%), sorbitol (6%)[b]	12 mos.
500 mg./3.5 Gm.	Polyethylene glycol 400 (85%), polyethylene glycol 4000 (15%)	18 mos.
330 mg./7.5 Gm.	Jelene® (water absorption base)[a]	12 mos.
330 mg./7.5 Gm.	Polyethylene glycol 400 (85%), polyethylene glycol 4000 (15%)	12 mos.

[a] The Jelene ointment bases are products of the Research Products Corporation.
[b] Contains 0.2% methyl paraben and 0.05% propyl paraben as preservatives.

TABLE IV.—STABILITY OF NEOMYCIN IN HYDROPHOBIC OINTMENT BASES AT 23°

Neomycin Base Content	Composition of Ointment	Stability Data: Time, Mos.	Stability Data: Activity Loss
3.3 mg./Gm.	White petrolatum	34	None
3.3 mg./Gm.	Lanolin (1%), white petrolatum (99%)	34	None
3.3 mg./Gm.	Lanolin (1%), white petrolatum (98%), water (1%)	24	None
3.3 mg./Gm.	White petrolatum (96%), lanolin (3%), benzocaine (1%), and methyl and propyl parabens	18	None
3.3 mg./Gm.	Jelene® (non-water absorbing)	15	None
330 mg./3.5 Gm.	Liquid petrolatum (75%), white petrolatum (25%)	18	None
330 mg./3.5 Gm.	Pennsylvania Refining Base No. 4-120	18	None
330 mg./3.5 Gm.	White petrolatum (50%), 2% aluminum monostearate in peanut oil (50%)	15	None[b]
330 mg./3.5 Gm.	White petrolatum (50%), 2% aluminum monostearate in sesame oil (50%)	18	None[b]
330 mg./7.5 Gm.	Lanolin anhydrous	12	None
330 mg./7.5 Gm.	Lanolin hydrous	12	90%
500 mg./3.5 Gm.	Falba®[a] (75%), polyethylene glycol 400 (25%)	12	None
330 mg./7.5 Gm.	Lanolin anhydrous (50%), water (50%)	12	15%
330 mg./7.5 Gm.	White petrolatum (50%), peanut oil (50%)	12	None
330 mg./7.5 Gm.	Falba®[a] (75%), peanut oil (25%)	12	None[b]

[a] Falba, an oxycholesterin absorption base, is the product of Pfaltz and Bauer, Inc.
[b] Oil separation noted.

In addition to the data presented in Table I, a series of stability studies have been conducted with relatively concentrated aqueous solutions of neomycin sulfate and neomycin hydrochloride (50 to 400 mg. of base per ml.). In these experiments additives such as phenol (0.25%), sodium citrate (1–3%), and sodium metabisulfite (0.1–0.3%) and combinations thereof were tested. Samples were stored at 23 and 45° for periods ranging from twelve to eighteen months without loss of potency. It was observed that combinations of sodium citrate and sodium metabisulfite (with or without phenol) offered the greatest protection against discoloration of the solutions.

SUMMARY

A study of the data accumulated to date indicates that neomycin possesses excellent stability properties in a variety of vehicles for long periods of time at normal and elevated temperatures.

In dilute aqueous solutions, neomycin salts were found to be stable up to two years at room temperature with excellent color stability. At higher temperatures for two years, there are indications that the use of antioxidants and buffers in the *p*H 6–7 range may aid in retarding the development of color of the solutions.

In concentrated aqueous solutions, neomycin salts were found to be stable for one to two years at normal and elevated temperatures in the presence of various combinations of antioxidant and buffering agents. The inclusion of these additives retards the development of color in the solutions.

Aqueous nasal preparations, mouth washes, and tinctures were found to be stable for at least one year at room temperature.

TABLE V.—STABILITY OF NEOMYCIN IN COMBINATION WITH OTHER ANTIBIOTICS IN OINTMENT BASES

Neomycin Base Content	Content of Other Antibiotics	Composition of Base	Stability Data at 23°: Time, Mos.	Activity Loss: Neomycin	Activity Loss: Other
3.3 mg./Gm.	Cryst. penicillin G potassium, 10,000 u/Gm.	White petrolatum	24	None	None
3.3 mg./Gm.	Cryst. penicillin G potassium, 10,000 u/Gm.	White petrolatum (85%), sesame oil (15%)	24	30%[a]	None
3.3 mg./Gm.	Cryst. penicillin G potassium, 10,000 u/Gm.	White petrolatum (97%), cholesterol (3%)	24	25%[a]	None
3.3 mg./Gm.	Cryst. penicillin G potassium, 10,000 u/Gm.	Falba® (85%), sesame oil (15%)	24	35%[a]	35%
3.3 mg./Gm.	Cryst. penicillin G potassium, 10,000 u/Gm.	White petrolatum (60%), Falba® (25%), sesame oil (15%)	24	None	15%
3.3 mg./Gm.	Cryst. penicillin G potassium, 10,000 u/Gm.	White petrolatum (97%), lanolin anhydrous (3%)	24	15%	15%
3.3 mg./Gm.	Cryst. penicillin G potassium, 10,000 u/Gm.	Jelene® (non-water absorbing)	24	None	None
3.3 mg./Gm.	Cryst. penicillin G potassium, 10,000 u/Gm.	Polyethylene glycol base, U. S. P.	24	None	Unstable
3.3 mg./Gm.	Bacitracin 500 u/Gm.	White petrolatum	20	None	10%
5.0 mg./Gm.	Bacitracin 500 u/Gm.	Polyethylene glycol 400 (60%), polyethylene glycol 4000 (40%)	13	None	Unstable
330 mg./3.5 Gm.	Cryst. penicillin G procaine, 100,000 u/3.5 Gm.	White petrolatum (50%), 2% aluminum monostearate in peanut oil (50%)	18	None	20%
500 mg./3.5 Gm.	Cryst. penicillin G procaine, 100,000 u/3.5 Gm.	Polyethylene glycol 400 (85%), polyethylene glycol 4000 (15%)	12	None	Unstable
330 mg./7.5 Gm.	Cryst. penicillin G procaine, 150,000 u/7.5 Gm.	Falba® (75%), peanut oil (25%)	12	None	20%
50 mg./7.5 Gm.	Cryst. penicillin G procaine, 300,000 u/7.5 Gm, bacitracin 2500 u/7.5 Gm.	Polyethylene glycol 400 (85%), polyethylene glycol 4000 (15%)	15	50%[a]	Penicillin, bacitracin unstable
50 mg./7.5 Gm.	Cryst. penicillin G procaine, 300,000 u/7.5 Gm., bacitracin 2500 u/7.5 Gm.	White petrolatum	15	30%[a]	None
50 mg./7.5 Gm.	Cryst. penicillin G procaine, 300,000 u/7.5 Gm., bacitracin 2500 u/7.5 Gm.	Falba® (75%), mineral oil (25%)	15	20%[a]	None
50 mg./7.5 Gm.	Cryst. penicillin G procaine, 300,000 u/7.5 Gm., bacitracin 2500 u/7.5 Gm.	White petrolatum (50%), peanut oil (50%)	15	20%[a]	No loss of penicillin; 25% loss of bacitracin

[a] In view of the data obtained above on the stability of neomycin, losses in neomycin activity of this order seem improbable. Some difficulties in assay have been encountered as a result of the necessity for inactivating other antibiotics during the assay procedure.

In both water-miscible and water-immiscible ointment bases, neomycin is stable for one to two years at room temperature. Similar results were noted for neomycin in combination with other antibiotics in oil suspensions and a variety of ointment bases. A notable exception is the marked instability of neomycin in hydrous lanolin.

Neomycin tablets and troches containing the usual excipients were stable for two years at room temperature and 56°.

Data have been presented to show the effect of autoclaving on dilute solutions of neomycin sulfate and of the effect of dry heat sterilization on neomycin sulfate in the dry state and in an ointment base.

TABLE VI.—STABILITY OF NEOMYCIN IN COMBINATION WITH OTHER ANTIBIOTICS IN OLEAGINOUS SUSPENSIONS

Neomycin Base Content	Content of Other Antibiotics	Composition of Base	Stability Data at 23°: Time, Mos.	Activity Loss: Neomycin	Activity Loss: Other
330 mg./10 ml.	Cryst. penicillin G procaine, 150,000 u/10 ml.	Peanut oil (90%), lanolin anhydrous (10%)	12	None	None
330 mg./10 ml.	Cryst. penicillin G procaine, 150,000 u/10 ml.	Peanut oil (70%), lanolin anhydrous (30%)	4	None	20%
500 mg./5 ml.	Cryst. penicillin G procaine, 150,000 u/5 ml.	Peanut oil (70%), lanolin anhydrous (30%)	3	None	None
5.0 mg./ml.	Cryst. penicillin G procaine, 10,000 u/ml., cryst. dihydrostreptomycin sulfate 5 mg./ml.	Peanut oil	6	None	None

TABLE VII.—STABILITY OF NEOMYCIN IN COMPRESSED DOSAGE FORMS

Neomycin Base Content	Content of Other Active Ingredients	Composition of Matrix, Gm./Tablet	Stability Data: 23°	Stability Data: 56°
16.7 mg./tablet	Bacitracin, 2500 u/tablet	Corn starch 0.15 Confectioner's sugar 0.25 Acacia 0.02 Magnesium stearate 0.005	At 19 mos., no loss of neomycin or bacitracin	At 19 mos., no loss of neomycin, 36% loss of bacitracin
167 mg./tablet	None	Lactose 0.10 Corn starch 0.10 Acacia 0.005 Magnesium stearate 0.005	At 21 mos., no loss	At 21 mos., no loss
167 mg./tablet	None	Corn starch 0.10 Confectioner's sugar 0.10 Acacia 0.01 Magnesium stearate 0.006	At 21 mos., no loss	At 21 mos., no loss
3.3 mg./troche	Benzocaine, 5 mg./troche	Acacia 0.05 Corn starch 0.15 Confectioner's sugar 0.25 Sodium saccharin 0.0001 Magnesium stearate 0.005 Oil of peppermint *q. s.*	At 18 mos., no loss	At 18 mos., 15% loss
2.5 mg./troche	None	Corn starch 0.15 Acacia 0.05 Confectioner's sugar 0.25 Magnesium stearate 0.0045	At 18 mos., no loss	Not tested

TABLE VIII.—DRY HEAT STERILIZATION OF NEOMYCIN SULFATE IN THE DRY STATE AND IN OINTMENT FORM

Sterilization Cycle: 10 Hours at 110°, Hot-air Oven

Sample	Test Product	Stability Data	Comments
NNW-5	Neomycin sulfate, dry powder	No loss in potency	Powder turned yellow
Neomycin ointment	White petrolatum 3.15 mg. base per Gm.	13% loss in potency	Ointment liquefied, darkened—redispersion of antibiotic necessary

TABLE IX.—EFFECT OF AUTOCLAVING ON SOLUTIONS OF NEOMYCIN SULFATE IN SEALED CONTAINERS (CONCENTRATION 3.25 MG. OF BASE PER ML.)

Duration of Autoclaving at 120°, Min.	Color[a] (Transmittancy, %)	Loss in Potency, %
0 (control)	98	0
20	96	0
40	90	0
60	82	14
90	70	10
120	61	10

[a] Measured on the Leitz Rouy-Photrometer at 445 mμ using a 10 × 10-mm. cell.

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Application of Chromatography in the Analysis of Acorn Oil*

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The unsaponifiable portion of acorn oil adsorbed on an alumina column was resolved into a hydrocarbon, a wax, a carotenoid pigment, and β-sitosterol by successive elution of the column with petroleum ether, carbon tetrachloride, benzene, and chloroform-alcohol, respectively. By fractional crystallization from an acetone solution at temperatures ranging from −10 to −50°, the saturated acids were separated from the unsaturated ones. The former were adsorbed on silicic acid and located on the extruded column by streaking with mercuric iodocyanide reagent. The divided column yielded palmitic and stearic acids. The unsaturated acids in acetone solution were adsorbed on alumina and subjected to frontal analysis at −70°. Oleic and linoleic acids were isolated from the eluates. Results of all separations were checked by application of standard procedures in fat analysis. Isolates from acorn oil were identified in the conventional manner.

THE CHEMICAL composition of acorn oil (*Quercus rubra*, L.) was reported by Monarca and Lynn (1). It was selected for this investigation simply because of the abundance of the nuts in the vicinity.

The isolation of sterols and pigments by chromatography has been reported frequently, however the literature offers few records of the resolution of the unsaponifiable portion of a fat by adsorption. An artificial mixture simulating unsaponifiable matter was eluted from a column with a series of solvents (2), and the success encountered in trial runs was adapted to the unsaponifiable portion of acorn oil, whereby a hydrocarbon, a wax, a sterol, and a pigment were isolated.

The separation of mixtures of fatty acids through the solubilities of their metallic salts and through the fractionation of their methyl esters are standard procedures. Adsorption techniques have been applied to a wide variety of artificial mixtures with varying degrees of success (3–8). The most successful separations on this basis are reported for either acids of different homologous series or for acids of a given series which, however, are not too closely related to one another. It seemed desirable, therefore, to attempt a chromatographic separation of the acids of acorn oil and to check the results by applying the lead salt-ether and the bromination procedures.

Reports in the literature suggest that chromatographic separation of fatty acids is difficult. Preliminary tests were performed on artificial mixtures of fatty acids so that the experiences gathered there might be applied to the natural mixture. The resolving power of a variety of adsorbent-indicator systems was tested against different combinations of myristic, palmitic, stearic, oleic, and linoleic acids, with no over-all success applicable to this problem. The adsorbent-indicator systems tested were: alumina-bromthymol blue (9), heavy magnesium oxide-phenol red (6), heavy magnesium oxide-phenolphthalein, silicic acid-bromcresol green (10), and silicic acid-RNH_4 indicator (10, 11).

The possibility of producing colored derivatives, through which the acids could be located on a colorless column, was explored. Treatment of the hydroxamates of stearic, palmitic, and myristic acids with ferric chloride (12) produced orange-red complexes which could not be resolved on a silicic acid column.

A quantitative separation of lauric, myristic, palmitic, and stearic acids was accomplished by streaking their extruded chromatogram (silicic acid) with mercuric iodocyanide reagent (13) and eluting the individual acids from the respective sections. This technique is described under the procedure for separating the saturated acids of acorn oil. The unsaturated acids were separable on alumina by a modification of the procedure described by DiModica and Rossi (14).

EXPERIMENTAL

The fixed oil used in this investigation was obtained from the acorns of *Quercus rubra*, L.[1] Fresh kernels contained 31.74% of water (15) and 8.79% of oil, extractable with low boiling petroleum ether. The oil content of the dried kernels averaged 13.00%. After extracting the oil on a large scale, it was freed from traces of solvent by bubbling nitrogen through it, while warming gently. It was stored under nitrogen, at refrigerator temperature. The

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[1] Identification by Prof. G. Safford Torrey, of the Botany Department.

oil was yellow in color and possessed a caramel-like sweetish odor, and a mild, nutty taste. A thin film spread on a glass plate remained liquid after eighteen days, indicating nondrying properties. Sp. gr. $\frac{25°}{25°} = 0.9175$; $n_D^{25°} = 1.4710$.

The chemical constants, determined by standard procedures, are listed in Table I.

TABLE I.—CHEMICAL CONSTANTS OF ACORN OIL

Sample	I	II
Acid value	1.63	1.57
Saponification value	195.18	195.24
Iodine number	97.51	97.26
Unsaponifiable matter, %	0.773	0.788
Titer test	14.8	14.8
Hehner value	94.1	94.4
Reichert-Meissl number	1.0	0.99
Acetyl value	5.1	5.0

Unsaponifiable Matter.—A 500-Gm. quantity of the oil was saponified by refluxing on a steam bath for two hours with a solution of 200 Gm. KOH in 3 L. of ethanol. After the alcohol was removed by distillation, the residue was dissolved in hot water. The unsaponifiable matter was extracted from the cooled soap solution with ether in the usual manner. Complete removal of the solvent left a residue weighing 3.7838 Gm. (0.756%). It was a yellowish-brown, semicrystalline mass, streaked with white deposits.

Chromatographic Separation.—A portion of the unsaponifiable matter weighing 0.2703 Gm. was dissolved[1] in 25 cc. of petroleum ether and adsorbed on a 2 cm. x 6.5 cm. column of adsorption alumina (Fisher Scientific Co., 80–200 mesh.). The column was successively eluted with 75 cc. each of petroleum ether, carbon tetrachloride, benzene, and chloroform containing 10% ethanol, in the order mentioned. The extracts were freed of their respective solvents, dried in a vacuum, and weighed (Table II).

To serve as a guide, a parallel test was run on an artificial mixture of 51.3 mg. paraffin, 24.6 mg. carotene and 50.6 mg. cholesterol. Petroleum ether eluted the paraffin; carbon tetrachloride removed nothing; benzene eluted almost all of the coloring matter and the alcoholic chloroform desorbed the sterol, together with a very small quantity of the pigment. Recoveries in this test were very efficient (Table II).

Examination of the Eluates.—The petroleum ether eluate of the chromatogram prepared from the unsaponifiable matter of acorn oil was a colorless liquid, $n_D^{25} = 1.4739$, b. p. 115° as determined in a micro boiling point tube. Its insolubility in concentrated sulfuric and in fuming sulfuric acids indicated a paraffin hydrocarbon. Its constants did not coincide with those of any hydrocarbon reported in the literature.

The unsaponifiable fraction of fixed oils is known in many instances to consist of a mixture of hydrocarbons, wax alcohols, sterols, waxes and pigments. The fact that carbon tetrachloride extracted nothing from the artificial mixture of hydrocarbons, sterol and pigment, but did yield an extractive from the natural mixture suggested the presence of a wax or, a wax alcohol in the latter. The material recovered from the carbon tetrachloride solution was a yellow-brown semisolid which did not respond to the Lieberman-Burchard test. Hydrolysis with amyl alcohol and hydrochloric acid resulted in the separation of needlelike crystals which gave a positive reaction to the Takahashi test (16) for aliphatic alcohols. Recrystallization from ethanol produced fine needles melting at 82°.

The mother liquor from the hydrolysis was evaporated, and the residue, dissolved in ether, was shaken with sodium hydroxide solution. The alkaline solution in turn was acidified and extracted with ether to isolate any free acids. The presence of the carboxyl group was demonstrated by the reaction of ferric chloride with the hydroxamate, as described by Feigl (12). From these results, it is apparent that the carbon tetrachloride did elute a wax from the chromatogram of the unsaponifiable matter. Unfortunately, the quantities isolated were so small as to allow for general identification only.

That portion of the unsaponifiable residue eluted

TABLE II.—CHROMATOGRAPHIC SEPARATION OF UNSAPONIFIABLE MATTER

	Paraffin		Carotene		Cholesterol	
Artificial Mixture	Added, mg.	Recovered, mg.	Added, mg.	Recovered, mg.	Added, mg.	Recovered, mg.
Petroleum ether	51.3	51.4	24.6	...	50.6	...
Carbon tetrachloride		...		...		...
Benzene		...		22.0		...
Chloroform-ethanol		...		...		53.9

Unsaponifiable Matter	Added, mg.	Recovered, mg.	Lieberman-Burchard Test
	270.3		
Insoluble residue[a]		18.7	−
Petroleum ether		35.1	−
Carbon tetrachloride		9.6	−
Benzene		5.4	−
Chloroform-ethanol		209.1	+
		Total 275.2	

[a] Identified as silicone grease.

[1] An insoluble residue weighing 18.7 mg. was identified as silicone grease.

with benzene was of a deep orange color, suggestive of the carotenoid pigments. This substance was taken up in 25 cc. of an acetone-petroleum ether solution and chromatographed on a 10-cm. column of Celite-magnesium oxide mixture (1:2) topped by a 3–5-cm. layer of anhydrous sodium sulfate. Elution was performed with 25 cc. of the mixed solvents, followed by 25 cc. of petroleum ether alone. The solutions were combined and evaporated in a vacuum. The residue, dissolved in chloroform, gave a color reaction identical to that of crystalline carotene when treated with a chloroformic solution of antimony trichloride (17).

Evaporation of the chloroform-ethanol eluate of the original chromatogram left a yellowish-white crystalline mass which was decolorized in hot alcoholic solution. Crystallization from hot aqueous alcohol gave lustrous platelets melting at 137–138°. They gave a positive Lieberman-Burchard test. An attempt was made to fractionate the sterol by adsorbing it from petroleum ether solution on alumina and eluting with the chloroform-ethanol solvent. Three fractions were collected, and the crystals recovered from all were shown to be identical to the original sterol, indicating that the compound was pure and that it was but a single sterol. The acetate, prepared from a 50.0-mg. sample, melted at 126.5–127°; the benzoate, prepared from a 63.2-mg. sample, melted at 146–147°. The melting point of the sterol and of its two esters identify it as β-sitosterol.

Fatty Acids.—After warming it gently to remove traces of ether, the aqueous soap solution from which the unsaponifiable matter had been removed was acidified with hydrochloric acid. The water-insoluble fatty acids separated as a white curd which formed a clear oily layer on the surface, when the mixture was heated. This was separated, washed thoroughly with hot water and filtered through dry paper. Drying was completed at 100° for twenty minutes.

Separation and Identification.—The combined fatty acids were resolved into the saturated and the unsaturated portions by the lead salt-ether method (18) and by low temperature fractional crystallization. The first method showed the mixture to consist of 21.8% saturated acids and 78.2% unsaturated acids. For the low temperature fractionation, a 7.5% acetone solution of the mixed fatty acids was exposed to temperatures which decreased in 10° increments from −10° to −50°. Six fractions of crystalline matter were collected, washed thoroughly with cold acetone, and recrystallized from petroleum ether. Fractions 1–3 consisted of stearic acid and fractions 4–6 consisted of palmitic acid. These were identified by the melting points of the free acids and of their *p*-bromophenacyl esters (Table III).

TABLE III.—SATURATED ACIDS ISOLATED BY LOW TEMPERATURE FRACTIONAL CRYSTALLIZATION

Fraction	Weight (Gm.[a])	Melting Point (° C.) Fatty Acid	Melting Point (° C.) *p*-Bromophenacyl Ester
1	1.1061	66–67	89–90
2	2.6525	66	89–90
3	0.4085	65	89–90
4	0.2060	60	85
5	0.8113	59–60	84–85
6	0.0656	59–60	84
	Total 5.2500		

[a] The sample of mixed fatty acids weighed 25.0000 Gm. The saturated acid component agrees very closely with that indicated by the lead salt-ether method.

Duplicate samples of the saturated acids were analyzed by the chromatographic procedure, using a streak reagent for locating the zones. Benzene solutions, approximately 0.01 *M*, were placed on a column of Merck's reagent grade silicic acid, 2 cm. x 20 cm., and development with benzene was conducted until the first trace of acid appeared in the eluate. The column was extruded and streaked with mercuric iodocyanide reagent (13). After standing for five to ten days, a red color developed at the site of the adsorbed acids. (In trial runs with known mixtures, the acids were found to be located in the order of increasing molecular weight, with the lowest member of the series located at the top of the column.) After sectioning, the acids were eluted with chloroform containing 5% ethanol. Two fatty acid fractions were obtained, one melting at 55–58° (lower zone) and the other at 50–51°. Each fraction was rechromatographed, and after elution, the residues having like melting points were combined and recrystallized several times from ethanol. Melting point determinations showed the presence of palmitic and stearic acids (Table IV).

TABLE IV.—CHROMATOGRAPHIC SEPARATION OF SATURATED FATTY ACIDS

	Sample No. I	Sample No. II
Wt. of Sample	117.3 mg.	102.0 mg.
Wt. of Fraction 1	25.0 mg.	21.5 mg.
Wt. of Fraction 2	94.0 mg.	79.8 mg.
m. p. of Fraction 1	62–63°	62°
m. p. of Fraction 2	67–68°	67–68°

The unsaturated fatty acids were isolated by two different procedures and identified. The lead salts soluble in ether were decomposed with hydrochloric acid in the usual manner. A 10% solution of the acids in anhydrous ether was brominated at −15°, until a reddish yellow color persisted. The excess of bromine was destroyed by the dropwise addition of β-amylene, and the solution was maintained overnight at −15°. No crystals separated, indicating the absence of linolenic acid hexabromide.

The ethereal solution was evaporated and the residue was extracted with boiling petroleum ether. This was refrigerated, whereupon linoleic acid tetrabromide crystallized out. It was crystalline at −20°, but liquid at higher temperatures. This corresponds to the observations of several investigators (19) who reported the existence of two or more isomeric tetrabromides, one of which is liquid.

Removal of the solvent from the mother liquor of the tetrabromide produced a brown oily residue which was debrominated with zinc dust in ethanolic solution. Following hydrolysis of the ethyl ester, oleic acid was isolated and identified by oxidation with alkaline permanganate to dihydroxy stearic acid. Oleic acid represented 65.7% and linoleic 34.2% of the unsaturated acids in acorn oil.

The residual acetone solution, from which the saturated acids had been removed by low tem-

perature fractional crystallization, contained the unsaturated acids. This was chromatographed, employing a modification of the procedure described by DiModica and Rossi (14). An aliquot portion of the solution was adsorbed on a 1 cm. x 15 cm. column of Fisher's adsorption alumina (80–200 mesh.), which was cooled to −50° by immersion in a dry ice-alcohol bath. The container for this bath, in which the chromatographic tube was supported vertically by the rubber stopper, was an inverted Erlenmeyer flask (4 L.) from which the bottom had been cut. The large opening facilitated the addition of dry ice to maintain low temperatures. The acids were washed through at −70° with acetone, under slight nitrogen pressure. Ten fractions of 30–40 cc. each were collected, the solvent for the last fraction being acetone containing 5% acetic acid. After removal of the solvent, the iodine numbers of the residues were determined by Niemierko's method (20), using the pyridine sulfate dibromide reagent suggested by Rosenmund and Kuhnhenn (21) (Table V). The iodine numbers for oleic and linoleic acids are 90.07 and 181.42, respectively, indicating the presence of these two acids in the appropriate fractions. This pattern was substantiated by a parallel run on a known mixture of oleic and linoleic acids.

TABLE V.—CHROMATOGRAPHIC SEPARATION OF UNSATURATED FATTY ACIDS

Fraction	Eluant, cc.	Weight (mg.)[a]	Iodine No.
1	30	78.9	158.0
2	30	208.8	181.8
3	30	44.3	95.0
4	30	27.8	98.3
5	40	23.4	92.1
6	40	16.0	90.6
7	40	17.1	...
8	40	23.1	90.5
9	40	15.9	...
	acetone + 5% acetic acid		
10	50	145.2	94.2
		Total 600.5	

[a] The aliquot taken represented a sample of 600.0 mg. of unsaturated acids.

SUMMARY

Results of this study show that chromatographic procedures can be applied successfully in the "total" analysis of a natural fat. All chromatographic methods employed were paralleled by standard procedures of fat analysis for the purposes of evaluation and reassurance.

The unsaponifiable matter yielded a hydrocarbon, a wax, β-sitosterol, and a carotenoid pigment.

The fatty acids isolated were identified as palmitic, stearic, oleic, and linoleic.

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A Comparative Study of Mineral Oil Separation in Pharmaceutical Emulsions*

By LAURENCE A. WASSON

Five commercial preparations were studied for oil separation under varying conditions, as in mixtures with synthetic intestinal and gastric juices, with varying amounts of bile salts, with fresh natural bile, and at varying temperatures and levels of *p*H. Other studies included separation and measurement of oil from these preparations when admixed with a digestive fluid prepared from fresh beef intestine. A laboratory sample of U. S. P. mineral oil emulsion was used as a control and Scarlet Red was used to delineate oil separation more clearly.

A CAREFUL search of the literature shows that while some papers have been written calling attention to oil separation from an emulsion, *per se* (1), or even when diluted, there has been little written concerning the separation of oil from an emulsion when in contact with the body fluids found within the intestinal tract. Due to the highly competitive market of today it is a matter

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of prime importance that each manufacturer be certain that his product is the finest that can be produced, and for that reason no expense is spared to evaluate thoroughly his product and run comparative tests on similar products in the same class.

The problem of oil separation in emulsions is of special importance in laxative type emulsions because this type is expected to mix with the feces without any attendant oil leakage, which, if occurring, would cause dissatisfaction to the user. In addition, under extremes of temperature encountered in the shipping and storing of these products, oil separation may sometimes occur. It was decided to compare five commercial emulsions of the mineral oil type with a laboratory sample of U. S. P. mineral oil emulsion, especially with regard to oil separation and to include in this study such other data as could be obtained from variations in *p*H, digestion time, centrifugation time, digestive formulas, storage conditions, and a comparison between oil separations in the presence of both natural and synthetic fluids.

EXPERIMENTAL

The emulsions[1] used in this study included, (A) an emulsion containing agar, tragacanth, acacia, albumin, glycerin, phenolphthalein, and mineral oil; (B) a colloidally dispersed mineral oil containing vitamin A; (C) an emulsion described simply as an "Aqueous Suspension of Mineral Oil"; (D) a mineral oil emulsion of "Brewer's Yeast"; (E) an Emulsion of Liquid Petrolatum, U. S. P. made by the Mortar Method; and (F) an "Emulsion of Mineral Oil with Irish Moss" (Chondrus).

Method.—To an accurately weighed 25.0-Gm. sample the other ingredients were added, as indicated in the tables, and sufficient digestive fluid was then added to bring the volumes to 50 ml.

Digestive Fluids.—The fluids mentioned in this study are similar to those used by other workers in the evaluation of the enteric coatings of tablets (2). The types used were:—(I) artificial gastric juice (Bauer's) (2); (II) artificial intestinal juice (New Type) (2); (III) artificial intestinal juice (Older Type) (3); and (IV) a natural intestinal juice extraction prepared as follows:—A section of intestine, approximately 18 inches long, was obtained from freshly slaughtered beef cattle and slit open longitudinally from the pyloric sphincter downward. This was sectioned transversely into small pieces and ground with sharp clean sand in a large mortar.

Further extraction with artificial intestinal fluid (New Type above), but having the pancreatin omitted, was carried out until a total volume of 500 cc. was collected. This was stored at 37° until used.

Other Digestants.—(V) *Bile salts.*—Concentrated bile salts were used in these experiments representing a tenfold concentration from fresh bile. (VI) *Steapsin.*—A purified grade of lipolytic enzyme was used. (VII) *Steer bile.*—Fresh steer bile was obtained from freshly slaughtered animals. It was found to have a *p*H of 6.95.

[1] All except (E) were commercial preparations.

PROCEDURE

Preliminary Digestion.—All digestions were carried out in special test bottles (Kimball Glass Co., #15066) of approximately 50-ml. capacity having the neck graduated from 0 to 100% in 2% intervals and having a capacity of 10 ml. in the graduated portion. A 25-Gm. sample of each emulsion was weighed out, the bile salts or other substances added as shown in the various tables, and the volume of the mixture then made up to 50 ml. with the digestive fluids. The mixtures were then stirred slightly and placed in the test bottles and transferred to the digestion bath. Digestions were carried out either in a water bath equipped with a thermoregulator or in an oven at 37° as indicated in the tables.

A special rack was constructed from aluminum sheet, to accommodate the six test bottles which were suspended by means of a swivel from an eccentric arm of a mechanical shaker in a constant temperature bath at 37°. The samples were shaken at a moderate rate with a combined vertical and rotary motion during the digestion periods indicated in the tables, thus imitating the natural peristaltic undulations.

Centrifugation for Separated Oil.—At the end of the period of digestion the bottles were removed from the shaker and centrifuged at 1500 r. p. m. for one-half hour, in all cases except where noted.

Delineation of Oil Phase.—Following the period of centrifugation a few milligrams of Scarlet Red dye were scattered onto the surface of the fluid in the test bottles and these mixtures allowed to stand overnight to allow completion of the solubility of the dye in any separated oil. Volumes of oil were read as accurately as possible but if the volumes were below 0.2 ml. as accurate an estimation as could be made was recorded. Any volume greater than the capacity of the graduated stem was transferred to a graduated cylinder for measurement.

In the following tables, unless otherwise noted, all digestions were carried out for a three-hour period with constant agitation in a water bath at 37° and were followed by centrifuging for one-half hour at 1500 r. p. m. The oil separation was then delineated by the addition of Scarlet Red dye to the separated oil.

TABLE I.—OIL SEPARATED FROM EMULSIONS IN THE PRESENCE OF SYNTHETIC GASTRIC AND INTESTINAL JUICES

Emulsion	Digestive Fluid Type I (*p*H 1.2)	Digestive Fluid Type II (*p*H 6.9)	Digestive Fluids Types I, III equal volumes (*p*H 6.7)
A	None	Trace	None
B	80%	Trace	None
C	0.2%	0.8%	0.2%
D	Sl. trace	Trace	Sl. trace
E	None	V. sl. trace	Sl. trace
F	None	V. sl. trace	None

TABLE II.—OIL SEPARATION FROM EMULSIONS IN THE PRESENCE OF SYNTHETIC INTESTINAL JUICES CONTAINING BILE SALTS AND OTHER SUBSTANCES

Emulsion	Digestive Fluid Type II + 0.1 Gm. Bile Salts[a]	Digestive Fluid Type II + 0.1 Gm. Bile Salts and 0.1 Gm. Steapsin	Digestive Fluid Type III + 0.3 Gm. Powd. Ox Bile (1:8) Ext. U. S. P.
A	Trace	Sl. trace	None
B	Trace	None	None
C	Trace	Trace	Trace
D	Sl. trace	None	Trace
E	Trace	Sl. trace	44.4%
F	Sl. trace	None	Trace

[a] All in this series were centrifuged an additional one-half hour but showed no additional separation of oil.

TABLE III.—OIL SEPARATED FROM EMULSIONS IN THE PRESENCE OF SYNTHETIC GASTRIC AND INTESTINAL JUICE TO WHICH FRESH BILE WAS ADDED

Emulsion	Digestive Fluid Type I + 0.3 Gm. Fresh Bile[a]	Digestive Fluid Type II + 0.3 Gm. Fresh Bile[b]
A	0.8%	Sl. trace
B	68%	None
C	1.6%	None
D	4%	0.4%
E	1.6%	Sl. trace
F	Sl. trace	Trace

[a] This series was digested six hours in water bath at 37°.

[b] This series was digested three hours in water bath at 37°.

TABLE III-A.—OIL SEPARATION FROM EMULSIONS IN THE PRESENCE OF SYNTHETIC GASTRIC JUICE AND BILE SALTS DIGESTED VARYING LENGTHS OF TIME

Emulsion	Digestive Fluid Type I	Digestion at 37°, —Water Bath, %— 30 Min.	60 Min.	90 Min.
E	+0.3 Gm. Bile Salts	0.2	0.4	0.8
F	+0.3 Gm. Bile Salts	0.32	0.8	1.6

TABLE III-B.—OIL SEPARATIONS FROM U. S. P. EMULSION AND CHONDRUS EMULSION IN THE PRESENCE OF SYNTHETIC GASTRIC JUICE AND VARYING CONCENTRATIONS OF BILE SALTS. ALL WERE DIGESTED FOUR HOURS, AT 37° IN A WATER BATH

Emulsion	Digestive Fluid Type I + 0.3 Gm. Bile Salts, %	Digestive Fluid Type I + 0.1 Gm. Bile Salts, %	Digestive Fluid Type I + 0.3 Gm. Powd. Ox Bile Ext. U. S. P., %
E	0.3	4.0	0.2
F	3.2	1.6	0.2

SUMMARY AND CONCLUSIONS

1. In the study of oil separation from six mineral oil emulsions, all but one showed very little oil separation when agitated with artificial gastric juice (*p*H of 1.2) for a period of three hours. The same emulsions showed very good stability in the presence of artificial intestinal juice (*p*H of 6.9) or in a mixture of equal volumes of both artificial gastric and intestinal juices. In the presence of synthetic intestinal juice the chondrus type emulsion appeared to be about as stable as the U. S. P. mineral oil emulsion and more stable than the others, while in the mixed juices it was slightly more stable than the U. S. P. emulsion.

2. Upon the addition of 0.1 Gm. of bile salts to the mixtures of the emulsions and synthetic intestinal fluid having a *p*H of 6.9, there was slightly less oil separation in most cases than when they were omitted. The same series, with an addition of 0.1 Gm. of steapsin, showed less oil

TABLE IV.—OIL SEPARATIONS FROM EMULSIONS IN THE PRESENCE OF AN EXTRACT OF FRESH BEEF INTESTINES. (DIGESTED THREE HOURS AT 37° IN WATER BATH)

Emulsion	Digestive Fluid IV Natural Intestinal Extract—No Pancreatin But 0.1 Gm. Bile Salts Added, %	Digestive Fluid IV Natural Intestinal Extract + 0.5% Pancreatin + 0.1 Gm. Bile Salts, %
A	0.2	V. sl. trace
B	V. sl. trace	None
C	0.8	4
D	0.4	Sl. trace
E	0.8	0.8
F	0.2	0.2

TABLE V.—OIL SEPARATIONS FROM EMULSIONS AT VARIOUS *p*H LEVELS. (THREE-HOUR INCUBATION IN OVEN AT 37°)

Emulsion	Digestive Fluid Type I + 1 *N* HCl to *p*H of 1.0	Digestive Fluid Type I + 0.5 *N* NaOH to *p*H of 2.0	Digestive Fluid Type I + 0.5 *N* NaOH to *p*H of 3.0
A	Sl. trace	V. sl. trace	V. sl. trace
B	3.0%	80%	29.6%
C	Trace	Sl. trace	Sl. trace
D	V. sl. trace	V. sl. trace	None
E	Sl. trace	Sl. trace	None
F	V. sl. trace	V. sl. trace	None

TABLE VI.—OIL SEPARATION FROM SIX EMULSIONS[a] STORED ONE WEEK AT 0° F., FOLLOWED BY THAWING AT ROOM TEMPERATURE AND SUBSEQUENT CENTRIFUGING FOR ONE-HALF HOUR AT 1500 R. P. M.

Emulsion	Storage at 0° F.	Sep. Oil, %
A	1 week	2.4
B	1 week	76
C	1 week	44
D	1 week	72
E	1 week	33.6
F	1 week	None

[a] Digestive Fluid Type I + 0.3 Gm. Fresh Bile.

separation than did those without this addition. In another series, to which 0.3 Gm. of powdered ox-gall extract was added to the older type intestinal fluid (*p*H of 7.85), most emulsions were stable but the U. S. P. emulsion showed a large amount of oil separation.

3. The addition of 0.3 Gm. of fresh bile to the series, using the newer type intestinal juice, did not seem to give results materially different from those in which 0.1 Gm. of bile salts was used, although two cases showed more oil separation in the fresh bile series. It was further noted, in a comparative study of the U. S. P. and the chondrus type emulsions, that when no bile salts were present the amount of oil separated was roughly proportional to the time of digestion. However, when varying amounts of bile salts or substances containing them were added to the digestions in the synthetic gastric fluid somewhat erratic results were obtained which were difficult to evaluate.

4. Less oil appeared to be separated when bile salts were added to the natural intestinal extract without the addition of 0.5 per cent pancreatin than in a similar case in a series run in which 0.5 per cent pancreatin had been added.

5. When the *p*H level of synthetic gastric juice was raised from 1.0 to 2.0 and this was added to the emulsions only one emulsion showed any marked oil separation. This emulsion broke almost completely and, as the *p*H of the synthetic gastric juice was raised further from 2.0 to 3.0, it still was the only one to show any appreciable oil released from the emulsion. It is interesting to note that the most stable emulsions at a *p*H of 3.0 were the chondrus type and the U. S. P. mineral oil emulsion.

6. When the emulsions were stored for one week at 0° F. and then centrifuged after thawing the separation of oil was marked varying from an extreme of 76 per cent in the case of the "Vitamin A—Mineral Oil Dispersion," to *none* in the case of the chondrus type emulsion. Even the U. S. P. emulsion showed an appreciable amount of separated oil in this extreme test.

7. It is concluded that of the various types of mineral oil emulsions studied both in the presence of gastric juice, and intestinal juices both natural and artificial, at varying *p*H levels and even after extreme freezing temperatures for one week, the chondrus type of emulsion was the most permanent.

8. However, since the stability of certain emulsions seemed to vary widely with only slight changes of *p*H, it seems that further investigations might well be carried out in regard to this factor, and that the foregoing experiments might be considered preliminary in nature.

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An Improved Assay for Magnesium Citrate Solution*

By DONALD D. ABBOTT† and LOUIS A. REBER

The present official methods of analysis for magnesium citrate solution are involved and tedious. The assays for total citric acid and for magnesium oxide have been simplified without sacrificing accuracy to any degree. Use has been made of a cation exchange resin to convert the total acid to a titratable form. Citric acid may then be calculated directly. The magnesium oxide has been determined by a titration employing disodium ethylenediaminetetraacetate. The combination of these two procedures for the analysis of magnesium citrate solution allows a great saving of time with an accuracy equal to or better than that achieved using the official methods.

THE PRESENT OFFICIAL ASSAY of U. S. P. magnesium citrate solution (1) consists of a determination of *Minimum of Total Citric Acid* as well as an *Assay of Magnesium Oxide*. The citric acid determination consists of the precipitation of calcium citrate, ignition to calcium oxide, followed by a residual acidimetric titration. The assay for magnesium oxide is a gravimetric procedure based on the precipitation and weighing of magnesium hydroxyquinolate. Each of these official methods, although satisfactory as a means of assay, has its shortcomings. The method for citric acid requires a great deal of manipulation resulting in a long and tedious procedure making its use awkward as a routine laboratory procedure. The gravimetric procedure for the assay of magnesium oxide is simple and accurate but nevertheless time-consuming.

This paper presents methods for the determination of magnesium as well as citric acid which are

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improvements over the present official methods. Each of these proposed methods results in a considerable reduction of time required and has proved to be equally accurate and even more precise than the present U. S. P. method. In the procedures developed in this study, magnesium is determined volumetrically by the use of the disodium salt of ethylenediaminetetraacetic acid, while total citric acid is titrated with alkali after liberation from its salts by the use of an ion exchange resin.

Disodium ethylenediaminetetraacetic acid (disodium salt of EDTA) and the ion exchange resins have been receiving ever increasing use in the field of analytical chemistry. The use of the disodium salt of EDTA for the determination of magnesium was introduced by Schwarzenbach (2) for the determination of magnesium and calcium in water; the method is similar to that suggested by Mattocks and Hernandez (3) for the assay of calcium products and used by Griffenhagen, Pfisterer, and Sloth (4) for the assay of tablets containing calcium. For the determination of citric acid, use has been made of an ion exchange resin to replace the cations of the solution by hydrogen ions, thus making possible a titration of the total acid with standard alkali.

EXPERIMENTAL

Magnesium Determination

Reagents.—(*a*) *Solution of Disodium Salt of EDTA.*—Dissolve 10 Gm. of the disodium salt of EDTA in sufficient water to make 1 liter. This solution should be stored in a pyrex bottle to ensure its stability.

(*b*) *Standard Magnesium Sulfate Solution.*—Prepare an aqueous solution of $MgSO_4 \cdot 7H_2O$ so that 1 ml. of solution is equivalent to 1 mg. of magnesium. This solution may be standardized by any suitable method for the determination of magnesium. In our work the hydroxyquinoline gravimetric procedure was employed.

(*c*) *Buffer Solution.*—Dissolve 67.5 Gm. of ammonium chloride and 570 ml. of concentrated ammonium hydroxide in enough water to make 1 L. of solution.

(*d*) *Indicator Solution.*—Inhibited Versenate Indicator.[1]

Standardization of the Solution of the Disodium Salt of EDTA.—Transfer exactly 25 ml. (equal to 25 mg. of magnesium) of standard $MgSO_4$ solution to a 250-ml. beaker and add exactly 50 ml. of the solution of the disodium salt of EDTA. Stir and allow to stand ten minutes, add 20 ml. of buffer solution and titrate the excess disodium salt of EDTA with standard $MgSO_4$ solution using two drops of indicator solution. The end point of the titration, a change from a dark blue to a wine-red color, is readily observed when the beaker is held above a white surface. Calculate the amount of magnesium (in mg.) equivalent to 1 ml. of the solution of the disodium salt of EDTA.

Determination of Magnesium Content of Magnesium Citrate Solution.—Transfer to a 100-ml. volumetric flask exactly 10 ml. of magnesium citrate solution which has previously been freed from excessive carbon dioxide by repeated pouring. Dilute to mark and mix thoroughly. Transfer exactly 25 ml. of this dilution (equivalent to 2.50 ml. of sample) to a 250-ml. beaker, add exactly 50 ml. of the solution of the disodium salt of EDTA and allow to stand ten minutes. Add 20 ml. of buffer solution and titrate the excess disodium salt of EDTA with standard $MgSO_4$ solution using two drops of indicator solution. From the magnesium equivalent to 50 ml. of solution of the disodium salt of EDTA subtract the amount of magnesium contained in the standard $MgSO_4$ solution used in the titration. The difference, multiplied by 40, is the magnesium contained in 100 ml. of the sample. The magnesium content multiplied by 1.658 gives the equivalent amount of MgO.

The residual titration of magnesium was resorted to after considerable experimentation with a direct titration procedure. The end point of the direct titration proved quite difficult to observe and inconsistent results were obtained using this method. This is in agreement with the findings of Mattocks and Hernandez (3).

Total Citric Acid Determination

Reagents and Apparatus.—(*a*) *Tenth-normal Sodium Hydroxide.*

(*b*) *Ion Exchange Resin*—Amberlite® IR-120 (H) analytical grade.[2]

(*c*) *Ion Exchange Column*—It is suggested that the ion exchange column have the following characteristics: 300 mm. in length, 8 mm. in diameter, one end constricted to an aperture of 4 mm. and packed with a small wad of glass wool. Five grams of resin are introduced into the column by means of a funnel inserted into the upper end and washed with water until firmly and evenly packed. The rate of flow of water through the properly prepared column should be 80 to 100 drops per minute. A column prepared in this manner can be used three times in the procedure described below before it is regenerated or discarded.

PROCEDURE

Transfer exactly 25 ml. of the same dilution as used in the determination of magnesium to a 150-ml. beaker and heat to just below the boiling point. Pour the hot solution through the exchange column, collecting the effluent in a 250-ml. flask. Wash the beaker twice with 25-ml. portions of hot water, collecting the washings in the same flask. Allow the resulting solution to cool and titrate with tenth-normal sodium hydroxide, using phenolphthalein as indicator. Each milliliter of tenth-normal sodium hydroxide is equivalent to 7.005 mg. of citric acid.

In experimenting for the best means of achieving the ion exchange the addition of varying amounts of resin directly to dilutions of prepared magnesium citrate solution, of known citric acid content, was tried. The results obtained in titrations of solutions

[1] Versworth Chem. Co., Framingham, Mass.

[2] Rohm and Haas, Philadelphia, Pa.

TABLE I.—COMPARISON OF METHODS FOR MAGNESIUM DETERMINATIONS
(GRAMS OF Mg PER 100-ML. SAMPLE)

U. S. P. XIV Method	Proposed Method
0.940	0.938
0.940	0.937
0.938	0.938
0.940	0.938
Av. 0.940	Av. 0.938
S. D. = 0.001	S. D. = 0.0005

TABLE II.—RECOVERY OF ADDED MAGNESIUM BY TITRATION WITH DISODIUM EDTA
(GRAMS OF Mg PER 100-ML. SAMPLE)

Amt. Added	Amt. Present	Amt. Found
None	0.940	0.940
0.050	0.990	0.988
0.100	1.040	1.040
0.150	1.090	1.094
0.200	1.140	1.138

TABLE III.—COMPARISON OF METHODS FOR CITRIC ACID DETERMINATION
(GRAMS OF CITRIC ACID PER 100-ML. SAMPLE)

U. S. P. XIV Method	Proposed Method
9.23	9.24
9.20	9.26
9.26	9.24
9.24	9.25
Av. 9.23	Av. 9.25
S. D. = 0.022	S. D. = 0.009

TABLE IV.—RECOVERY OF ADDED CITRIC ACID BY ION EXCHANGE METHOD
(GRAMS OF CITRIC ACID PER 100-ML. SAMPLE)

Amt. Added	Amt. Present	Amt. Found
None	9.25	9.25
0.250	9.50	9.49
0.500	9.75	9.74
0.750	10.00	10.00
1.000	10.25	10.24

treated in this manner gave recoveries of citric acid ranging from 95 to 98%. It was for this reason that the present method using the column, rather than the batch technique, has been employed.

RESULTS AND DISCUSSION

Magnesium Determination.—In order to test the suitability of the suggested method for the determination of magnesium, a sample of magnesium citrate solution was analyzed, for magnesium content, by both the U. S. P. XIV method and the method described herein. The results are shown in Table I. To test further the reliability of the suggested method known quantities of magnesium were added to the original magnesium citrate solution and analyses repeated. The recovery of these added amounts of magnesium are given in Table II.

As is shown in Tables I and II the results obtained using the suggested method for magnesium are comparable, in accuracy and precision, to the present U. S. P. method. At the same time, a considerable reduction in the time required to complete each individual assay is achieved.

Total Citric Acid Determination.—Analyses of magnesium citrate solution were carried out, using both the U. S. P. XIV method for *minimum of total citric acid* and the proposed method for *total citric acid*. A comparison of the results obtained using these two methods is presented in Table III. The reliability of the proposed method was ascertained by adding known amounts of citric acid to the original magnesium citrate solution. The results showing the amounts of citric acid recovered are presented in Table IV. The ion exchange method for the determination of total citric acid is shown to give results comparable to those obtained using the official method while it is capable of even better precision.

SUMMARY

1. The proposed methods for the determination of magnesium and total citric acid content of magnesium citrate solution produce accurate and precise results comparable with those obtained using the present official U. S. P. methods.

2. The methods presented herein allow for a considerable reduction in time and eliminate the unsatisfactory aspects of the current official methods.

3. It should be realized that the proposed ion exchange method for the determination of total citric acid is not specific for that acid. By means of this method, all cations are replaced by hydrogen ions and are titrated with alkali, regardless of the acid radicals with which they are combined.

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Compressed Tablets: Internal Flow of Granulation During Compression*

By ALLAN M. RAFF†, ANGEL S. ARAMBULO‡, ALFRED J. PERKINS, and DWIGHT L. DEARDORFF

Tablets were compressed on a power driven single-punch tablet press from several types of granulations. The hopper was filled completely and compression proceeded continuously until the granulation was exhausted. The tablets were collected in small groups in order of compression and weighed. Statistical quality control charts were prepared for weight, hardness, and compressional pressure. During the first period of compression the weight usually increased, but sometimes decreased, depending upon the nature of the granulation. During the latter part of the period of compression there was a material decrease in weight regardless of the granulation employed. The internal flow and segregation of granulation in the hopper is believed to contribute to this effect. Preliminary evaluation of concomitant measurements of compressional pressure and tablet hardness indicate a possibility of a correlation of pressure with weight and hardness.

THE STUDY OF MIXING and with it the phenomena of segregation, stratification, and the internal flow of granular material of solid-solid systems has been of interest to such industries as fuel (1, 2), paint (3), soap (4), ceramics, mining (5), metallurgy (5), food (6), drug (7, 8), and cosmetic (9), where it is an ever-present problem.

However, in general, little experimental work of a quantitative nature has been done on this subject (10–12). In the tablet industry few articles have been published to indicate any examination of the reasons for mechanical separation of particles in a granulation, or to what extent the separation varies when the different physical properties of the granules are varied. Recent work in this laboratory suggests that some internal flow of granulation occurred during compression of tablets even when using relatively homogeneous material (13). Statements have been made that the uniformity of tablet mixtures could be modified during compression due to mechanical vibration of the tablet press (7).

Flow of granulation in the hopper of a tablet press is of a complex nature. It has been said to involve a consideration of the following factors: (*a*) *Voidage.*—The percentage of voids may be expressed by the formula: % voids = 100 − 100 (bulk density/absolute density) (14). The importance of voidage will be appreciated from the fact that it is through the voids that the fine particles migrate (14, 15).

(*b*) *Bulk density and absolute density.*—Bulk density is influenced by occluded air, size and shape of particles, coefficient of friction between particles, and absolute density (10, 11, 14, 16). The bulk density of the closest packing of a mixture of large and small granules is greater than the bulk density of the closest packing of either large or small granules, except in the case where there is a very small portion of either large or small granules in the mixture (2). Since bulk density is a measure of voidage, and since most workers apparently feel that the voidage must be kept to a minimum, it seems probable that for a given type of granulation there will be an optimum voidage, and therefore an optimum bulk density, that gives free, even flow of material in the hopper, but does not give too much segregation. It would seem that this optimum bulk density would perhaps approach the absolute density. If the granulation is composed of particles of different absolute densities the heavier will seek the bottom (2, 10, 11).

(*c*) *Particle shape.*—Round smooth particles seek the bottom and jagged or polyhedral particles seek the top (11). The flow of fines relative to the flow of coarse particles is, to a very large extent, governed by void content, which in turn, is influenced by the regularity in particle shape (11, 14, 16).

(*d*) *Particle size.*—Coarse particles tend to rise to the top of a granulation and fines tend to settle to the bottom. As the size ratio is increased the small particles slip through the voids between the large particles (7, 10, 17).

(*e*) *Packing and flow of particles.*—A granulation in a hopper constitutes a form of packing. As the granulation moves down the conical hopper it is distorted in shape and this distortion

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The authors gratefully acknowledge suggestions on statistical analysis of data made by Dr. H. C. Batson of the University of Illinois, College of Medicine.

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leads to expansion or dilatation of the granulation. A study of the mechanism of the flow of particles in granular beds has shown that the deformation of a packing leads to nonuniformity in the distribution of voidage and uneven flow of fine materials (1, 2).

(*f*) *Formation of agglomerates.*—Agglomeration in powder mixtures is intensified as the particle size is reduced due to the increased intermolecular surface attraction. Materials which tend to pack into small volume when in fine powder form show a definite tendency to agglomerate when mixed with other materials of a larger particle size. A high degree of agglomeration will result in relatively little segregation, all other factors being equal (7, 9, 17).

(*g*) *Flow of material from the hopper.*—Experiments have shown that the free flow of granular materials in a hopper is irregular and asymmetrical (1, 2, 18).

The purpose of this work was to investigate, by use of statistical quality control charts for weight, the internal flow of granulation and segregation (unmixing) in the press hopper during compression of several tablet granulations. A parallel study of compressional pressures and tablet hardness was begun.

The Control Chart

A control chart may be described as a graphic presentation of test data in such a manner that the variability of all the results is compared with the average variability within (arbitrary) small groups of the test results. The chart is said to show evidence of "control" when there is no more variation throughout the entire set of results than corresponds, statistically, to the average variation within the (arbitrary) small groups (19).

In the usual control chart this is shown by the position of the plotted points with respect to the horizontal lines normally called "control limits." In these charts each point represents the average value or the range of the measured characteristic of a selected number of items referred to as a subgroup. These values are plotted in sequence. A solid line gives the grand average ($\bar{\bar{X}}$) of the measured characteristic or the average range ($\bar{R}$) of the measurements of the characteristics of the lot. The control limits are also shown by horizontal lines. The upper and lower control limits for averages are calculated using the formulas: $CL_{\bar{X}} = \bar{\bar{X}} \pm A_2\bar{R}$.[1] The upper control limit for range is computed from the formula: $UCL_R = D_4\bar{R}$[1] and the lower limit when used by: $LCL_R = D_3\bar{R}$.[1]

The purpose of using control charts in this investigation is to demonstrate the effects of segregation on several tablet properties. Due to the nature of the data it was deemed advisable to modify slightly the usual control chart in order to make clear the phenomena taking place. To use any one of several possible formulas, based upon the values of the entire lot, for estimating three standard deviations of the mean ($3\,s_{\bar{X}}$) one obtains limits based only on the variation within subgroups. There is no allowance in these formulas for variation between subgroups. This means that these formulas are valid when the process under consideration is in control. When a situation exists where the process is not in control, as is the case in the experiments under study here, these formulas cannot be used in the calculations to determine the true process variability. The lack of control was shown by the downward trend of the mean values, $\bar{\bar{X}}$, while the ranges, R, remained relatively in control and application of analysis of variance indicated that the values did not follow a single normal distribution.

In order to emphasize the effects of segregation the limits in these experiments were based upon the formula: $CL_{\bar{X}} = \bar{\bar{X}}_{10} \pm A_2\bar{R}_{10}$ using only the first 10 subgroups in each experiment. It is assumed that these subgroups reflect only the inherent variability of the process before the effects of segregation have an opportunity to become evident. It is true that within this interval of the first 10 subgroups there may be more variation between subgroups than in other portions of the run. This is due to the fact that all values, including those of the first tablets compressed, are recorded because of the nature of the experiment. In normal production these initial values would have been discarded and no record made of them. However, in these experiments the purpose was not one of production. The variation between subgroups can be attributed to the "settling" of the granulation in this part of the run but within-group variation is nominal. To use different sections in the several runs for an estimate of the process variability was considered too subjective to be advisable.

While it is understood and appreciated that a sound statistical quality control procedure calls for a minimum of 25 subgroups upon which to base control limits it was deemed advisable to use 10 in these experiments. This conforms to what is termed a "bare minimum."

Another advantage of basing control limits on just those early values which reflect only inherent variability is that the process would start in a state of control and remain there until the effects of segregation become evident. Since the weight of the first tablets compressed is arbitrarily chosen as the nominal weight, it is important that the estimate of $\bar{\bar{X}}'$, (true universe average) for this portion of the run be centered on the nominal value.

Another modification made was in the elimination of the $\bar{\bar{X}}$ line in the control charts as an indication of the grand average weight of the batch. As there was no constant-cause system in operation throughout the period of measurement, it would be misleading to consider an $\bar{\bar{X}}$, based upon all measurements, as the grand average weight of one universe. Where measurements show lack of statistical control, the standard error of estimate of $\bar{\bar{X}}'$ has no meaning, as there is no one universe that has an average $\bar{\bar{X}}'$. The statement of a standard error in such a case tends to lead to a false confidence in the reliability of the average. Therefore, a value for $\bar{\bar{X}}$, designated as $\bar{\bar{X}}_{10}$, was calculated based upon the first 10 sub-

[1] A_2, D_3 and D_4 are constants, the values of which are determined by the number of samples in each subgroup (20).

groups; and this line, as well as the control limits, was extended across the chart to indicate the values that should have been obtained if the process had remained in control.

The subgroups were apportioned into sequential time intervals of five subgroups each in order to make the average values ($\bar{\bar{X}}_s$) for each sequential time interval be as sensitive as possible to the changes taking place in the subgroups.

In the control charts each point represents the average value of four tablets in a subgroup. These values were plotted in sequence. The lines connecting the points serve only to aid in visualization of the trend.

Using the same modifications as described for the $\bar{X}$ charts, control charts for ranges were also plotted to show the process variability as the control charts for averages show the process level. It should be noted that while the numerical values of the ranges may decrease in the latter part of the run they are increasing as a percentage of the tablet weight.

Table I contains average values ($\bar{X}$) and ranges (R) for the tablet weights, compressional pressure, and tablet hardness.

EXPERIMENTAL

Granulation fractionation was carried out using U. S. Standard sieves and a Ro-Tap Testing Sieve Shaker following, essentially, the method outlined in the ASTM Standards for refractory materials (21). Compression was carried out in the Stokes Model E single punch tablet press using $^{11}/_{32}$ in. punches. The machine was adjusted to a speed of approximately 100 tablets per minute. Enough of this material was used to fill the hopper of the machine completely (2–3 Kg.) and compression was allowed to proceed continuously (with one exception) until all the granulation had been compressed. This required from fifty to eighty minutes.

For the purpose of evaluation the first 4 tablets compressed were collected and assigned as the first

TABLE I.—TABLET WEIGHT, COMPRESSIONAL PRESSURE, AND HARDNESS AVERAGE VALUES ($\bar{X}$) AND RANGES (R)

		A		*B*		*C*		*B*		*B*	
		Lactose-Starch Weight, mg.		Sodium Chloride Weight, mg.		Lactose-Starch-Aspirin Weight, mg.		Sodium Chloride Pressure (Gauge deflections)		Sodium Chloride Hardness, Kg.	
Sequential Time Interval	Sample No.	$\bar{X}$	R	$\bar{X}$	R	$\bar{X}$	R	$\bar{X}$	R	$\bar{X}$	R
1	1	255.1	5.4	418.2	15.6	299.7	30 6	6.2	1.5	12.7	3.5
	2	242.8	4.6	413.4	20.0	305.9	19.0	6.1	1.6	14.6	3.7
	3	260.8	6.2	421.2	11.6	327.2	24.4	6.8	1.0	14.5	8.1
	4	252.9	6.6	431.7	15.0	277.2	11.8	7.8	1.4	14.2	5.3
	5	254.4	10.4	429.8	23.8	290.6	23.2	7.9	2.2	14.9	3.9
2	6	254.0	4.4	424.2	18.4	288.7	14.2	7.2	1.5	14.1	5.7
	7	258.9	5.0	430.4	14.0	299.3	16.8	7 6	1.5	15.1	8.8
	8	256.3	4.2	426.0	10.2	302.4	23.6	7.1	1.3	14.2	6.5
	9	254.4	4.2	425.2	2.0	285.8	38.2	7.2	0.3	14.7	2.4
	10	258.8	3.2	425.2	8.8	291.2	24.4	7.4	0.8	14.8	2.9
3	11	254.9	6.8	421.7	10.6	287.3	15.6	6.8	1.0	15.9	5.7
	12	257.0	8.2	422.3	19.6	276.2	7.2	6.9	2.0	14.0	2.9
	13	263.7	7.4	424.9	22.4	293.6	16.0	6.8	1.7	15.8	4.0
	14	259.2	2.0	415.8	10.0	297.4	31.6	6.2	1.2	14.0	2.3
	15	267.0	5.4	416.6	12.0	287.4	13.0	6 3	1.3	13.8	4.9
4	16	267.5	6.0	433.2	68.6	282.5	24.4	8.1	7.3	14.5	9.1
	17	267.9	7.2	424.1	8.8	280.4	11.4	7.0	0.5	13.5	3.2
	18	265.7	6.4	411.1	14.8	270.6	7.4	5.7	1.1	14.6	3.6
	19	263.7	9.8	418.3	24.4	277.8	14.4	6.1	2.4	14.4	3.7
	20	268.2	5.2	414.1	7.8	274.4	22.6	6.0	0.5	16.4	2.8
5	21	270.9	1.2	412.9	16.6	273.7	12.2	6.1	1.4	17.5	2.3
	22	266.9	4.2	406.5	19.0	287.0	21.2	5.3	1.5	16.8	5.9
	23	268.6	11.0	408.9	20.2	270.4	10.2	5.4	1.5	12.9	8.1
	24	271.5	4.8	405.7	19.6	277.3	8.2	5.2	1.5	12.6	2.5
	25	265.8	11.4	396.4	4.4	282.6	17.8	4.4	0.6	10.4	3.4
6	26	261.3	3.8	394 2	16.4	264.6	19.0	4.2	1.2	12.5	6.1
	27	256.6	6.4	391.4	11.4	258.8	9.4	3.9	0.7	12.9	8.2
	28	252.4	9.4	417 3	59.6	242.7	22.4	5.9	5.2	13.8	6.6
	29	261.2	4.4	388 6	20.0	...	...	4.0	0.8	12.6	2.0
	30	263.2	2.0	403.8	17.4	...	...	4.8	1.6	12.7	2.6
7	31	251.2	6.4	403.2	26.2	...	...	4.5	2.0	12.8	9.0
	32	255.1	20.0	392.0	22.6	...	...	3.8	1.6	9.8	5.5
	33	...	...	402.4	58.4	...	...	4.8	4.2	11.0	12.0
	34	...	...	410.2	48.4	...	...	5.5	3.7	13.6	12.0
	35	...	...	396.2	89.0	...	...	4.4	6.0	11.5	15.5
8	36	...	...	400.7	22.8	...	...	4.4	1.3	13.3	7.0
	37	...	...	377.7	38.2	...	...	3.2	2.0	7.9	5.9
	38	...	...	352.8	44.6	...	...	2.2	1.2	4.4	6.3
	39	...	...	329.5	82.0	...	...	1.5	2.7	4.2	9.3
	40	...	...	313.9	33.0	...	...	1.1	0.9	1.5	3.1
9	41	...	...	318.6	45.8	...	...	1.3	0.6	1.0	1.0

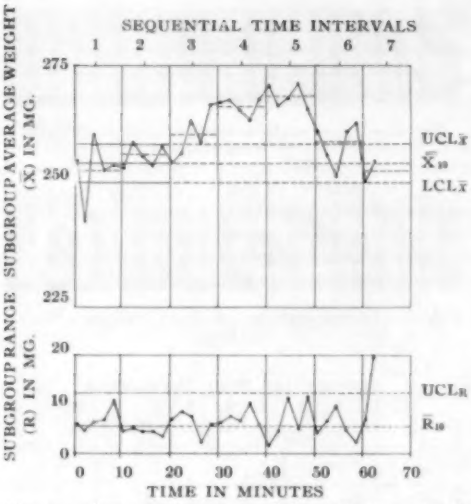

Fig. 1.—Control chart for weight of lactose-starch tablets.

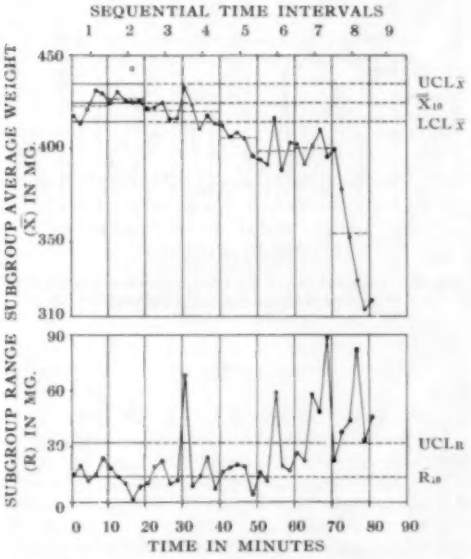

Fig. 2.—Control chart for weight of dyed sodium chloride tablets

subgroup. Subsequently four tablets were collected every two minutes. The last four tablets compressed were designated as the last subgroup. In some instances imperfect tablets had to be weighed to obtain values for these last subgroups. The tablets were weighed individually, on a Roller-Smith Balance, to 0.2 mg.

As an aid to following the weight trend, average weights were computed for each sequential time interval. These are indicated in the figures by intermittent lines.

Figures 1, 2, and 3 illustrate the general types of results obtained. Figure 1 deals with granulation *A*, a lactose-starch granulation made with a starch paste binder, and magnesium stearate and talc as

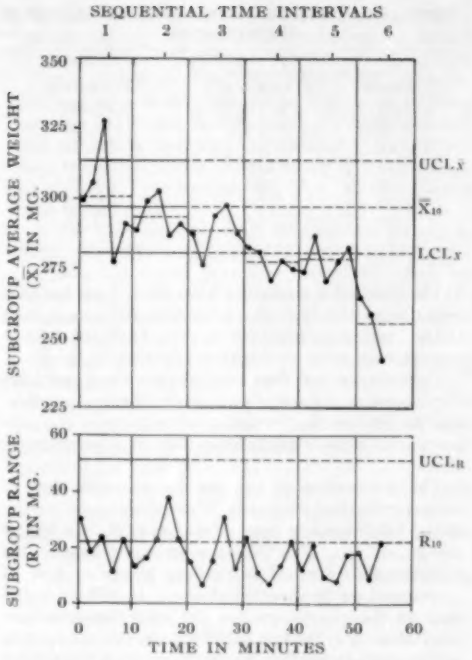

Fig. 3.—Control chart for weight of lactose-starch-aspirin tablets.

lubricants. Here the weight increased, passed through a maximum, and then decreased.

Figure 2 deals with granulation *B*, a sodium chloride granulation which was prepared by mixing equal parts by weight of granules which passed a No. 10 screen (2,000 μ) and were retained on a No. 12 screen (1,680 μ), and granules which passed a No. 45 screen (350 μ) and retained on a No. 50 screen (297 μ). The smaller particles had been dyed in order to follow their relative movement. In this run the weight remained relatively constant, increasing and then decreasing slightly during the first half of the run; for the remainder of the run there was a rapid decrease.

Figure 3 shows the results for granulation *C*, a mixture of 1,500 Gm. of lactose-starch granulation and 300 Gm. of aspirin powder. The lactose-starch granulation passed a No. 10 screen and was retained on a No. 50, the distribution of particle sizes being as shown in Table II. The aspirin passed a No. 70 screen (210 μ) and was retained on a No. 100 (149 μ). The aspirin was mixed with the lactose-starch granulation just prior to compression. In this run there was a rapid decrease in weight almost immediately and it continued to decrease except for one slight reversal.

These experiments seem to indicate that when one-half to one-quarter of the total granulation remains in the hopper the tablet weights begin to drop rapidly, leading to the conclusion that the die is being less completely filled due to a lack of fines in the mixture remaining in the hopper. This hypothesis was checked by an incomplete run using undyed granulation *B* in which the press was stopped when the run was about 85% completed and the granulation removed and sieved.

TABLE II.—PARTICLE SIZE OF LACTOSE-STARCH GRANULATION

Screen Size		
Passed	Retained	Percentage
10	12	12.86
12	14	16.14
14	16	26.91
16	18	13.00
18	20	11.21
20	25	7.03
25	50	12.85

The residual granulation from the hopper (no material from the feed shoe was included) contained coarse and fine particles in the ratio of 1.88:1 respectively, compared with the original ratio of 1:1. It should be noted that any breakdown of particles during compression due to hopper vibration, or during the final sieving, would tend to increase the proportion of smaller particles rather than to decrease them.

The movement of the smaller particles due to hopper vibration was also followed by examination of the tablets made from granulation *B*. In Fig. 4 the granulation *B* tablets are arranged in order of compression from left to right in groups of four to correspond to the control charts. It will be noted that as the run progresses the color becomes less marked until in the latter 10% of the run the tablets are colorless indicating a greatly reduced proportion of the small particles.

Figure 4 shows the upper surfaces of the tablets and Fig. 5 shows the lower surfaces of analogous tablets. The same trend of reduction in color is evidenced in both sets of tablets. However, for any given subgroup, the percentage of colored fines is always greater in the lower surfaces than in a comparable set of upper surfaces, hence the tablets are not homogeneous.[2] Note that in the next to the last series of tablets only the lower surfaces show any trace of colored fines while the upper surfaces are white. Therefore, with this granulation containing two groups of particles of widely separated size, stratification has taken place during the filling of the die and in the limited period of time after the filling and prior to compression. It would seem that this type of relative movement or unmixing could result under certain conditions in a significant concentration or localization of a tablet ingredient in the lower part of a tablet. This effect, coupled with the concomitant dilution effect in the upper part of the tablet might lead to undesirable results, such as difficulties in compression or during storage.

Exploratory experiments have been carried out to determine the relationship between compressional pressure, tablet weight, and hardness. The results of the pressure and hardness tests on the tablets of granulation *B* are given here as they give further insight into the phenomena of the internal flow and segregation of a granulation in a hopper.

Compressional pressures were measured for each of the granulation *B* tablets reported on above. Pressure was measured by means of a Baldwin Strain Gage SR-4 C-19 attached to a standard upper punch using a Brush Analyzer and oscillograph system (22–24) following substantially the method of Brake (25).

Figure 6 gives the compressional pressure data for granulation *B* tablets for which weights are plotted in Fig. 2. The pressures are expressed in terms of lengths of deflections obtained on the oscillograph. The tablets were collected in such a manner that it was possible to make individual comparisons of weights and pressures. A close correlation was ob-

[2] The authors gratefully acknowledge the suggestion of Mr. V. C. Campbell of Warren-Teed Products Co., that this phenomenon might be observed under certain conditions.

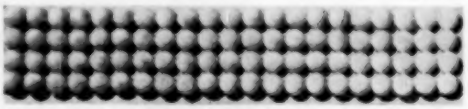

Fig. 4.—Upper surfaces of dyed sodium chloride tablets

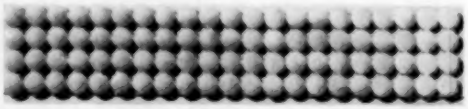

Fig. 5.—Lower surfaces of dyed sodium chloride tablets.

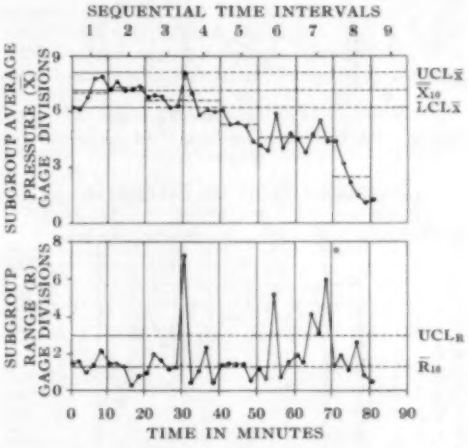

Fig. 6.—Control chart for compressional pressure of dyed sodium chloride tablets.

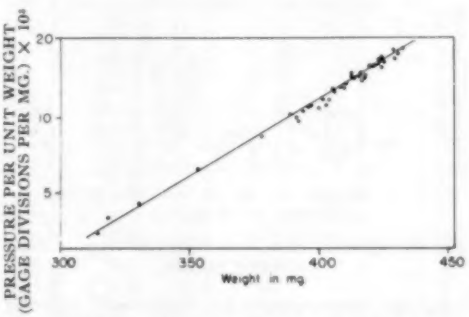

Fig. 7.—Compressional pressure per unit weight vs. weight for dyed sodium chloride tablets.

served between the weights and compressional pressures of specific tablets, as well as between the sequential time interval averages.

Figure 7 shows the relationship found for this granulation between pressure per unit weight and weight.

Hardness measurements were carried out on these same tablets using a Strong-Cobb tablet hardness device, modified to use line air-pressure and a variable-bleed device for control. Figure 8 shows a control chart for hardness values. It can be seen that it exhibits a trend quite similar to those of the pressure and weight charts.

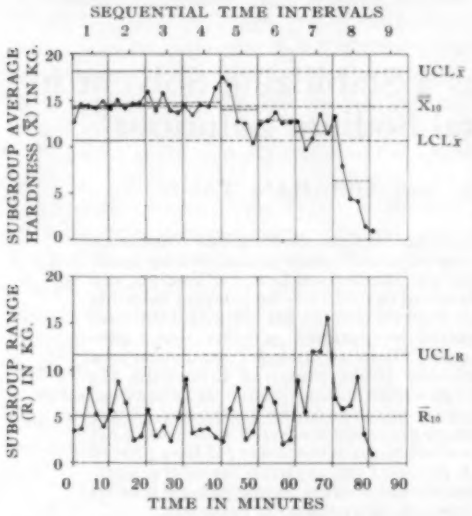

Fig. 8.—Control chart for hardness of dyed sodium chloride tablets.

DISCUSSION

It will be noted that in first part of the run the weight may remain relatively constant, increase, or decrease, apparently depending upon the characteristics of the granulation employed, while in the latter part of the run there is a substantial decrease in weight in every case. It is possible to obtain results in which the weights will gradually increase, pass through a maximum, and then in the latter part of the run become lighter due to the depletion of fines. This result is shown in Fig. 1. However results may be obtained in which the weights during the first part of the run remain relatively constant and then begin to decrease rapidly, as shown in Fig. 2; or as exemplified by the experiment illustrated in Fig. 3, the rapid decrease in weight may start almost immediately and continue with no significant reversals. Each type of trend has been observed in several other tests.

The above results are in agreement with results obtained in other fields on the study of the internal flow of particulate solids and with the theories worked out to explain the latter (1, 2).

The results obtained in these experiments reported upon support the production practice of keeping a hopper substantially filled at all times. However, developmental procedures using smaller batches do not always lend themselves to such a program.

Some tablets are made by adding the active ingredient to a basic granulation just prior to compression. The purpose of running a basic granulation combined with aspirin "fines" was to approximate such conditions and to study the flow of aspirin "fines" relative to the flow of the coarser basic granulation.

It has been demonstrated that there is an appreciable flow of fines relative to that of the coarse particles. It seems that any procedure which involves the addition of an active ingredient in the form of a fine powder to a relatively coarse granulation should be subjected to careful study.

Results of studies in other fields indicate that in order to avoid a fast flow of fines through coarser particles one should not have too wide a disparity in particle size, and the active ingredient should be of such a size as to fill substantially the average void spaces of the basic granulation (7).

The experiments reported upon here confirm the usual belief that it is important to check the tablet weights most closely during the first and last quarters of the run.

In order to obtain a rapid control method for use in the production of large quantities of tablets, it would seem that the tablet weight and hardness versus compressional pressure should be studied and the possibilities of correlating the tablet characteristics with the easily measurable operating characteristic (pressure) determined. Where a high degree of correlation is found, continuous monitoring of production would then be possible using a strain sensitive device and a suitable measuring instrument (oscillograph or even a simple ammeter).

The above method would have an added advantage besides its rapidity and accuracy in that if a relationship is found to exist between compressional pressure and tablet weight or hardness one could ascertain three values from one reading.

SUMMARY

1. Statistical quality control charts for tablet weight, hardness, and compressional pressure were used as an aid to the study of the internal flow of a granulation in the single punch tablet press hopper during compression.

2. The phenomena were enhanced by starting with a full hopper and operating until the supply of granulation was exhausted.

3. Under these conditions the tablets compressed during the first part of the run were substantially heavier than those produced in the latter part. It is believed that this was due to the disproportionate amount of fines present in the tablets compressed during the first part of the run.

4. The movement of fine material to the die was followed by means of sequential tablet weights, changes in color, compressional pressures, and hardness, supplemented by a test utilizing sieving at an intermediate point.

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Polyethylene Glycol 400 as a Stabilizing Solvent in Parenteral Pentobarbital Sodium Solutions*

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The stability of 15 per cent pentobarbital sodium in aqueous solutions containing 2 per cent benzyl alcohol and concentrations of polyethylene glycol 400 up to 60 per cent has been determined. The effects of *p*H, methods of heat sterilization, and the presence of 10 per cent alcohol were observed periodically on samples stored at room and at elevated temperatures. Results indicate that the *p*H influences the concentration of polyethylene glycol 400 required for optimum stability. At a concentration of 30 per cent of this glycol and at a *p*H of 10, aseptic formulation of a stable product is possible. Where autoclaving is the preferred procedure, the inclusion of 10 per cent alcohol is required to avoid discoloration. It has proved possible to prepare formulations containing 60 per cent of the glycol and 10 per cent alcohol at a *p*H at low as 8, in contrast to presently marketed solutions at *p*H 9.5 to 10 prepared with various glycols; these solutions at the lower *p*H have proved stable after sterilization by autoclaving. A proof of the applicability of the ultraviolet absorption method of assay of pentobarbital sodium in the presence of its decomposition products and other solution components is presented.

THE PREPARATION of stable solutions of barbiturates suitable for parenteral administration has long been a problem to the drug industry. Since the barbiturates themselves are insoluble in water, attempts have been made to dissolve them with the aid of compound solvents such as diethylamine, dimethylethylcarbinol, and ethylaminoformate, urethanes and amides of the lower fatty acids, and mixtures of alcohol, glycerin, and glycols.

More successful have been the attempts to prepare solutions of the sodium salts of barbiturates; however, these were found to be unstable due to alkaline hydrolysis, and several reports have appeared describing the decomposition of barbiturate salts in aqueous solution (1–4). Husa and Jatul (5) found that a lowering of the *p*H decreases the deterioration rate in aqueous solution, but not sufficiently to be practical, since this precipitates the free barbiturate. They suggested several stabilizers to retard decomposition on autoclaving.

A step in the right direction was accomplished when propylene glycol was introduced as a solvent for barbiturate salts; however, such solutions are known to acquire a light amber color on heating. Several proprietary products have been offered containing various concentrations of propylene glycol as the solvent, but the use of this glycol in parenteral solutions has recently been noted as open to question due to its possible toxic effects (6). The painful tissue damage due to the intramuscular injection of propylene glycol has been reported (7).

The relative safety of the polyethylene glycols as solvents for parenteral solutions has been established through extensive toxicological and physiological studies (8–10) and it has been shown that polyethylene glycol 400 (PEG 400) was relatively free from the marked tissue irritation heretofore shown by such vehicles as propylene glycol (7).

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Barbiturate salts in aqueous solutions of polyethylene glycol have been tested for activity and toxicity, and it has been demonstrated that these solutions show approximately the same potency and toxicity as aqueous solutions but produce less tissue irritation (11).

Scope of the Investigation.—The purpose of this investigation was to determine the conditions under which PEG 400 could be used to prepare a stable, heat-sterilizable solution of pentobarbital sodium. Among the problems involved in the formulation of a stable solution of this type were the effects of concentration of active ingredient (pentobarbital sodium), concentration of solvent components (PEG 400, alcohol, water), bacteriostatic agent (2 per cent benzyl alcohol), sterilization methods (steam at 100° and at 121°), and *p*H (between the limits 8–10).

A literature search for methods of determining the extent of pentobarbital sodium decomposition revealed that the spectrophotometric method (12–14) appeared to be particularly suitable because of its rapidity and permissible use of small samples. However, the possible interference in absorbancy due to the presence of barbiturate decomposition products and solution components had to be determined before the method could be applied.

EXPERIMENTAL

Materials.—The materials used in this investigation were the following: Dehydrated Alcohol, N. F.; Benzyl Alcohol, analytical reagent; Hydrochloric Acid, analytical reagent; Pentobarbital Sodium, U. S. P.; Phenobarbital Sodium, U. S. P.; Polyethylene Glycol 400, U. S. P., Carbide and Carbon Chemicals Co.; Polyethylene Glycol E400, "Texas Production," Dow Chemical Co.; Water for Injection, U. S. P.; Ampuls, 10 ml., alkali-resistant glass, Jena; Vials, 10 ml., U. S. P. Type I, "Neutraglass," Kimble; Vial Stoppers, white rubber, No. 86, West.

Preliminary Stability Experiments.—Since the bulk of the literature reports dealing with barbiturate decomposition are limited to phenobarbital, it was considered desirable to make a comparative study of the rate of decomposition of aqueous solutions of pentobarbital sodium and phenobarbital sodium when subjected to the temperature of the autoclave. Fifteen per cent solutions of each were made up in freshly boiled and cooled distilled water, and three samples of each solution were sealed under nitrogen in 10-ml. ampuls and autoclaved at 121° for fifteen, thirty, sixty, and one hundred twenty minutes, respectively. All solutions showed precipitates, the amounts increasing progressively with time of autoclaving. The concentrations were determined spectrophotometrically by diluting 5 ml. of the samples, thoroughly shaken to suspend uniformly the precipitates, to 15 μg. per ml. At this concentration complete solution occurred. The solutions were adjusted to *p*H 10 with dilute sodium hydroxide. The absorbancy–wavelength plots for these solutions, using the Beckman Model DU Spectrophotometer in the ultraviolet range, clearly indicated that the solution of phenobarbital sodium is more extensively decomposed.

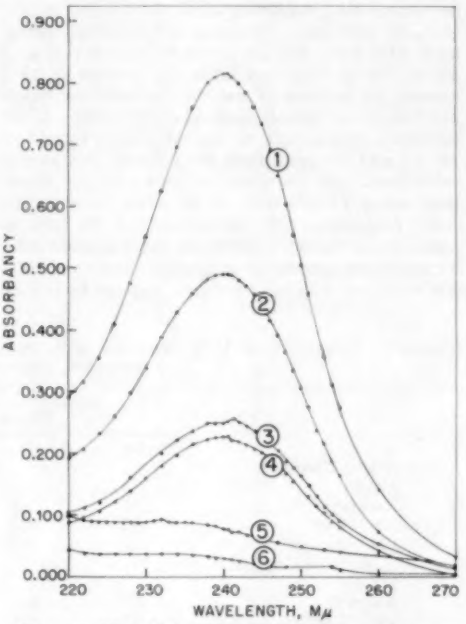

Fig. 1.—Ultraviolet Absorption Curves of Pentobarbital Sodium and its Thermal Decomposition Products. (1) Fresh solution, 20 μg./ml.; (2) Entire contents of 10-ml. ampul of (1) following 20 hrs. autoclaving at 121°; (3) Supernatant liquid of ampul treated as in (2); (4) Water-insoluble residue of ampul treated as in (2); (5) Crystalline sublimate obtained on drying precipitate recovered from first dilution in (2); (6) Nonvolatile residue from (5).

The approximate minimum *p*H limits, as a function of PEG 400 concentration, were determined by the dropwise addition of concentrated hydrochloric acid to a series of aqueous solutions containing 15% pentobarbital sodium and varying concentrations of PEG 400. Ten per cent alcohol was included in a similar series. The end point was taken as the *p*H where vigorous stirring failed to redissolve the cloud formed on adding a drop of acid.

The results revealed that it is not possible to prepare a 15% solution of pentobarbital sodium at a *p*H lower than 9.5 in the absence of 10% alcohol if the glycol content is less than 40%.

Further tests on this series of solutions were performed to determine their relative stability to autoclaving at 121° for twenty minutes. The viscosity of solutions containing 80% PEG 400 was particularly high, proving undesirable for injection. Color formation on autoclaving was noted in some solutions and tended to increase in intensity with increased glycol concentration and increased *p*H. As evidenced by the absence of precipitate formation, there was little destruction in the samples at

*p*H 10, but heavy precipitates were obtained with all solutions that were adjusted to *p*H 9.3 and *p*H 9.0, except those containing 40% PEG 400 and 10% alcohol at *p*H 9.3, and those containing 60% PEG 400 and 10% alcohol at *p*H 9.0 and 9.3. These results confirmed the following: (*a*) Only solutions containing 10% alcohol and at least 60% PEG 400 are stable to autoclaving at a *p*H as low as 9.0; (*b*) only solutions containing 10% alcohol and at least 40% PEG 400 are stable to autoclaving at a *p*H as low as 9.3; and (*c*) in the absence of 10% alcohol, no solution is stable to autoclaving below *p*H 9.5 at any concentration of PEG 400. Color formation was absent in the solutions adjusted to *p*H 9.3 and 9.0 using Dow PEG E400. Noticeable color formation was obtained under similar conditions using PEG 400 U. S. P. The cause of the color formation was determined to be due to impurities in the PEG 400 itself, since marked color formation was produced on heating samples of PEG 400 rendered alkaline by dilute sodium hydroxide and by phosphate buffers. The PEG 400 was found to contain traces of material that readily reduced acid permanganate in the cold. The presence of aldehydes was confirmed with 2,4-dinitrophenylhydrazine. It was concluded that the extent of color formation on heating alkaline mixtures of PEG 400 depended on the concentration of alkali, concentration and purity of the PEG 400, and the temperature and duration of exposure to elevated temperature.

Stability Study.—On the basis of the results obtained in these preliminary experiments, solutions of 15% pentobarbital sodium (w/v), in varying concentrations of PEG 400 (v/v), in the presence of 2% benzyl alcohol (v/v) were prepared, and measurements made of the effect on stability of *p*H (8–10), the presence of 10% alcohol (v/v), and modes of sterilization. The effect of storage of the various samples at room temperature for three and six months, and at 45° for three and six weeks was determined.

TABLE I.—STABILITY OF 15% PENTOBARBITAL SODIUM SOLUTIONS (2% BENZYL ALCOHOL PRESERVATIVE) IN 0 TO 20% POLYETHYLENE GLYCOL 400

	Initial		3 Weeks at 45°		6 Months at 25 ± 3°	
	Appearance[a]	Decomposition, %	Appearance[a]	Decomposition, %	Appearance[a]	Decomposition, %
Aqueous Control						
*p*H 10						
Aseptic.	A0	0.0	A2	2.5	A2	0.0
100°/1 hr.	A2	0.5	A2	3.2	A2	1.2
121°/20 min.	A3	5.0	A4	6.2	A5	1.2
10% Alcohol Control						
*p*H 10						
Aseptic.	A0	0.0	A2	3.3	A2	0.0
100°/1 hr.	A2	0.0	A2	3.2	A2	0.0
121°/20 min.	A2	2.2	A2	5.8	A2	0.7
10% PEG 400						
*p*H 10						
Aseptic.	A0	0.0	A2	2.5	A4	3.9[b]
100°/1 hr.	A0	0.5	A2	2.7	A2	2.3
121°/20 min.	A0	0.0	A2	2.7	A3	3.9
10% PEG 400 + 10% Alcohol						
*p*H 10						
Aseptic.	A0	0.0	A2	1.9	A4	13.1[b]
100°/1 hr.	A0	0.8	A2	2.5	A2	0.8
121°/20 min.	A0	1.0	A2	2.7	B2	1.0
20% PEG 400						
*p*H 10						
Aseptic.	A0	0.0	A2	1.3	A4	7.1[b]
100°/1 hr.	A0	1.7	A2	1.2	B2	1.2
121°/20 min.	A2	4.3	B3	4.5	C4	2.2
20% PEG 400 + 10% Alcohol						
*p*H 10						
Aseptic.	A0	0.0	A0	0.3	A0	0.0
100°/1 hr.	A0	0.0	A0	1.0	B0	0.0
121°/20 min.	B0	0.8	C0	2.3	C0	0.5
*p*H 9.5						
Aseptic.	A0	0.0	A0	0.7	B4	6.6[b]
100°/1 hr.	A0	1.7	A0	2.0	B0	0.0
121°/20 min.	A0	1.7	C0	2.3	C0	0.3

[a] *Key*
Color:
A—Colorless (no change from original).
B—Very pale yellow.
C—Pale yellow.
D—Yellow. See Table II.
E—Dark yellow to amber. See Table II.

Clarity:
0—Clear.
1—Very faint precipitate.
2—Light precipitate.
3—Medium precipitate.
4—Heavy precipitate.
5—Very heavy precipitate.

[b] No correspondence between the spectrophotometric assay and the amount of precipitate formed was observed. Spectrophotometric examination of these precipitated decomposition products showed them to consist essentially of free pentobarbital. This may possibly indicate that, when made by the aseptic procedure, solutions contain large molecular aggregates which act as nuclei for the formation of crystalline deposits on prolonged storage. Heat sterilization appears to obviate this formation.

TABLE II.—STABILITY OF 15% PENTOBARBITAL SODIUM SOLUTIONS (2% BENZYL ALCOHOL PRESERVATIVE) IN 30 AND 60% POLYETHYLENE GLYCOL 400

	Initial		3 Weeks at 45°		6 Months at 25 ± 3°	
	Appearance[a]	Decomposition, %	Appearance[a]	Decomposition, %	Appearance[a]	Decomposition, %
30% PEG 400						
*p*H 10						
Aseptic.	A0	0.0	C0	1.6	C0	1.0
100°/1 hr.	C0	0.8	E0	1.7	D0	3.6
121°/20 min.	D0	3.1	E0	4.1	D0	3.5
*p*H 9.5						
Aseptic.	A0	0.0	B0	1.3	B4	1.5[b]
100°/1 hr.	A0	1.0	C0	2.3	C0	2.6
121°/20 min.	C0	2.6	C0	3.5	D0	1.7
30% PEG 400 + 10% Alcohol						
*p*H 10						
Aseptic.	A0	0.0	A0	1.6	B0	0.2
100°/1 hr.	C0	0.8	B0	2.0	C0	0.2
121°/20 min.	C0	2.5	C0	0.5	D0	1.0
*p*H 9.5						
Aseptic.	A0	0.0	A0	0.0	A0	0.0
100°/1 hr.	A0	0.3	A0	0.0	C0	0.8
121°/20 min.	A0	0.5	C0	1.0	C0	0.8
60% PEG 400						
*p*H 10						
Aseptic.	A0	0.0	D0	0.8	D0	0.5
100°/1 hr.	C0	0.5	E0	1.2	E0	0.3
121°/20 min.	D0	1.0	E0	2.2	E0	1.0
*p*H 9.5						
Aseptic.	A0	0.0	C0	0.7	B0	0.0
100°/1 hr.	B0	0.5	D0	1.0	E0	0.0
121°/20 min.	C0	0.3	E0	0.8	E0	0.3
60% PEG 400 + 10% Alcohol						
*p*H 10						
Aseptic.	A0	0.0	D0	0.8	C0	0.5
100°/1 hr.	B0	0.0	E0	1.1	D0	0.5
121°/20 min.	C0	1.8	E0	2.4	D0	0.6
*p*H 9.5						
Aseptic.	A0	0.0	C0	0.0	B0	0.0
100°/1 hr.	A0	0.7	D0	0.2	B0	0.0
121°/20 min.	B0	0.5	E0	0.0	D0	0.0
*p*H 9.0						
Aseptic.	A0	0.0	C0	0.3	A0	0.0
100°/1 hr.	A0	0.0	D0	0.3	B0	0.0
121°/20 min.	B0	0.3	D0	0.0	B0	0.0
*p*H 8.0						
Aseptic.	A0	0.0	A0	0.0	A4	23.8[b]
100°/1 hr.	A0	0.3	A0	0.0	A0	0.2
121°/20 min.	A0	0.0	C0	0.2	A0	0.2

[a] See footnote (*a*), Table I.
[b] See footnote (*b*), Table I.

Each series was prepared in bulk, subdivided for adjustment to the specified *p*H, filtered through a sintered-glass crucible, filled under nitrogen into 10-ml. vials, and stoppered. The vials were divided into groups for immediate testing, steam sterilization at 100° for one hour, and autoclaving at 121° for twenty minutes. A series was prepared aseptically to serve as a control. Representative samples of the heat-sterilized and aseptically prepared series were set aside for immediate testing, sterility testing, and storage.

Sterility tests were performed on samples of each series using the U. S. P. method (15). In no case was there any indication of bacterial contamination.

The samples were examined visually, the *p*H determined with the Beckman Model H2 *p*H meter, and assayed spectrophotometrically by diluting filtered aliquots one million fold in distilled water previously adjusted to *p*H 10–10.5 with sodium hydroxide. The absorbancy, at *p*H 10–10.5, was determined using the Beckman Model DU spectrophotometer at 240.5 mμ and 0.77 mm. slit width in 1.000-cm. silica cuvettes, corrected for minor differences and absorbancy of the solvent components of the samples.

Data for the series containing 40 and 50% PEG 400 were omitted, since the results of the investigation showed no outstanding deviation from the 30% PEG 400 series. Only minor changes in *p*H were noted following autoclaving or steam sterilization and storage. The drop in *p*H on heat sterilizing by either process is of the order of 0.2–0.3 unit for the 0 and 10% PEG 400 series and, generally, not more than 0.05 unit for all others. The results of the stability study are summarized in Tables I and II.

Observations on Stability Study.—Increasing concentrations of PEG 400 have the effect of increasing

the stability of pentobarbital sodium, but at the same time make the solutions more prone to color formation, especially at the higher *p*H values. Solutions containing PEG 400 in concentrations below 30% with no alcohol, and below 20% with 10% alcohol are unstable under all conditions, while those containing concentrations above 30% are stable, but are highly prone to color formation on exposure to heat, unless the *p*H is lowered sufficiently.

The inclusion of 10% alcohol improves each formulation by increasing the stability of pentobarbital sodium and decreasing the extent of color formation. Its main value is that it permits a lowering of the *p*H in solutions of higher PEG 400 concentration.

As the *p*H is lowered, there is a slight increase in stability of pentobarbital sodium, and there is a marked decrease in color formation. The *p*H can be effectively lowered only if 10% alcohol is included in formulations of high (50–60%) PEG 400 concentrations. Satisfactory solutions at *p*H 10 can be produced if the PEG 400 concentration is kept at 20–30% and 10% alcohol is included. Such solutions are of sufficiently low viscosity to permit sterilization by filtration.

Both methods of sterilization, steam at 100° for one hour, and at 121° for twenty minutes, are effective and, under suitable conditions of *p*H and PEG 400 concentration, cause little destruction. Autoclaving may be employed for solutions containing 60% PEG 400 and 10% alcohol at a *p*H below 9 (preferably at *p*H 8 or slightly below). At higher *p*H values, the process may be used only where the PEG 400 concentration does not exceed 30%; otherwise, there is marked color formation.

In certain instances, trace sediment formation was noticed shortly after preparation in otherwise stable solutions. For solutions not destroyed by autoclaving, the following procedure was found to be successful in preventing the formation of such sediments: (*a*) autoclaving the solution in bulk at 121° for thirty minutes, (*b*) storage under refrigeration (5–8°) for forty-eight hours, (*c*) filtration in the cold, (*d*) aging at 45° for three weeks, (*e*) filtration followed by filling under nitrogen into containers, and (*f*) autoclaving at 121° for twenty minutes to sterilize.

Validity of Spectrophotometric Method of Assay.—For the purpose of determining exactly what influence the decomposition products of pentobarbital sodium have on the absorbancy data, the following procedure was carried out:

A 20% aqueous solution of pentobarbital sodium was prepared and 10-ml. aliquots transferred to each of three ampuls and sealed under nitrogen. These were autoclaved at 121° for a total of twenty hours. The *p*H before autoclaving was 9.75 and 9.25 after autoclaving. In all cases, the samples were dissolved with the aid of small quantities of alcohol and sodium hydroxide and the final dilution, at *p*H 10, contained the equivalent of 20 μg. per ml. of drug. Clear solutions resulted. Spectrophotometric data on the following samples or fractions were recorded: (1) fresh solution, (2) entire contents of one autoclaved ampul, (3) supernatant liquid of one autoclaved ampul, (4) water-insoluble residue from (3), (5) crystalline sublimate obtained, on drying at 90° for six hours, a precipitate recovered from the first dilution in (2), and diluted to 20 μg. per ml., (6) nonvolatile residue from (5).

The absorbancy–wave-length plots are given in Fig. 1. It is apparent that curves (5) and (6), representing decomposition products, differ considerably from that of pentobarbital sodium and show no maximum peak. These two plots are of solutions reconstituted to be of approximately the same weight concentration as fresh solutions of pentobarbital sodium, and their absorbancies dwindle to insignificance in the proportions that such decomposition products appear in ordinary solutions to which the method may be applied.

A further examination of the insoluble residue (4) was made by washing it first with distilled water, and second with 0.1 *N* NaOH. A comparison of the absorbancy spectra of solutions of these was made. The results indicate that considerable alkali-soluble material had been present in the deposit. The curve of the water-washed residue, upon solution, was typical of that of pentobarbital, corroborating the belief that the major constituent of the deposits that result on the thermal decomposition of aqueous solutions of pentobarbital sodium is indeed free pentobarbital itself. Similar observations on phenobarbital were reported by Nielsen (2) and Bailey (4).

Further proof of the application of the spectrophotometric method for determining pentobarbital sodium in the presence of its decomposition products was given by the corresponding results obtained spectrophotometrically and by a modification of the argentimetric potentiometric method described by Mattocks and Voshall (16). The modified method consisted of replacing the Fisher Senior Titrimeter with the Beckman Model G *p*H meter, and determining the end point as the first point at which the voltage reaches its final lowest value, disregarding any overvoltage which remains constant at the end of any determination; this overvoltage was found to vary from one determination to another. By this method, the pentobarbital sodium used was determined to be 98.95% pure.

The method was applied to three samples containing 15% pentobarbital sodium, 60% PEG 400, 10% alcohol, 2% benzyl alcohol which had been adjusted to *p*H 8 with HCl and had been prepared by (*a*) aseptic formulation, (*b*) steam sterilization at 100° for one hour, and (*c*) autoclaving at 121° for twenty minutes, all followed by six weeks' storage at 45° and three weeks at room temperature. The titrations were performed on ether extracts of the samples to avoid interference due to chloride in the samples. A comparison of the results of these titrations with the values obtained spectrophotometrically shows agreement within 0.7%.

The conformity of solutions of pentobarbital sodium to the Beer-Lambert law was established through the linearity of a concentration-absorbancy plot for solutions ranging from 5–25 μg. per ml. The specific absorbancy at *p*H 10, corrected for the known pentobarbital sodium purity, E (1%, 1 cm.) at 240.5 mμ, is 396.

The absorbancy of a 2% aqueous solution of benzyl alcohol, diluted one million fold, was determined at the above wave length and *p*H and was found to be zero. The absorbancies of solutions of PEG 400 were determined similarly at concentrations corresponding to one million fold dilutions

of 10, 20, 40, 60, 80, and 100% solutions. The corresponding absorbancies were a constant 0.006 ± 0.001. The same results were obtained on diluting solutions containing 10% alcohol and 2% benzyl alcohol, even after the mixtures were autoclaved, indicating that it was possible to apply a standard correction for the absorbancies of the nonbarbiturate components of the solutions.

The dependence of the absorbancy of pentobarbital sodium solutions on the *p*H of the medium was determined by adjusting a solution to various *p*H values and measuring the absorbancies. A plot of the absorbancy *vs.* *p*H showed a linear region in the *p*H range 10–11, proving the constancy of the absorbancy in the *p*H range 10–10.5 chosen for the assays.

CONCLUSIONS

1. The spectrophotometric method is applicable to the assay of pentobarbital sodium and phenobarbital sodium in the presence of their decomposition products and the solution components: benzyl alcohol, polyethylene glycol 400, ethyl alcohol, and sodium chloride.

2. The minimum *p*H attainable in 15% solutions of pentobarbital sodium containing up to 60% polyethylene glycol 400 is 9.5. Below this *p*H, solutions are destroyed by autoclaving. The inclusion of 10% alcohol permits a lowering of the *p*H to below 8 at 60% polyethylene glycol 400 concentration.

3. Color formation in solutions of pentobarbital sodium containing polyethylene glycol 400 increases as the concentration of polyethylene glycol 400 and the *p*H is raised. The color formation is due to a reaction of the polyethylene glycol (or impurities in it) with alkali, and can be eliminated by lowering the *p*H to 8 or the glycol concentration to 20%. Color formation is independent of the presence of pentobarbital sodium.

4. Solutions of 15% pentobarbital sodium show little chemical decomposition in various concentrations of polyethylene glycol 400 when subjected to autoclaving or steam sterilization, followed by storage for six weeks at 45° or six months at room temperature. Optimum stability is shown by solutions containing 60% polyethylene glycol 400 and 10% alcohol at a *p*H of 8 or slightly below. Solutions containing 20% polyethylene glycol 400 and 10% alcohol at *p*H 10 are stable, but should not be heat-sterilized nor stored at elevated temperatures. Solutions containing 30% polyethylene glycol 400 and 10% alcohol at *p*H 9.5 are also stable and may be sterilized, with caution, by autoclaving.

5. Unless these solutions are properly aged and then filtered before packaging they may develop a faint sediment shortly after preparation.

6. The following formulation of a stable pentobarbital sodium injection is suggested:

A solution is made containing 150 Gm. pentobarbital sodium, 100 ml. injection water, 600 ml. polyethylene glycol 400, 105 ml. alcohol (U. S. P.), and 20 ml. benzyl alcohol; it is adjusted with hydrochloric acid to *p*H 8 and made up to one liter with injection water. The bulk solution is autoclaved at 121° for thirty minutes. It is then stored under refrigeration (5–8°) for forty-eight hours, and filtered while still cold through a fine sintered glass funnel. It is then aged for three weeks at 45° and again filtered through fine sintered glass. It is filled under nitrogen into vials which are then sealed and autoclaved at 121° for twenty minutes.

If it is desired to prepare a solution of lower polyethylene glycol content, the above procedure should be modified by using only 200 ml. polyethylene glycol 400 and adjusting the *p*H to 10 just before making up to volume. The autoclaving steps, both of the bulk solution and of the filled containers, are eliminated in this alternate method. Instead the solution after aging at 45° is aseptically filtered under pressure through ultra fine sintered glass. It is then filled aseptically under nitrogen.

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A Pharmacological Study of 2-(Beta-hydroxyethylaminomethyl)-1,4-benzodioxane Hydrochloride*

By R. F. BANZIGER,† T. S. MIYA, and L. D. EDWARDS

The compound, 2-(β-hydroxyethylaminomethyl)-1,4-benzodioxane hydrochloride, abolishes or reverses the pressor action of epinephrine in the dog, rabbit, rat, and cat. With intravenous injections, dogs, cats, and rats showed vasodepression of from one to two hours duration. LD_{50} studies show that this compound is relatively nontoxic in rats, rabbits, and dogs but initial intravenous injections into cats were highly toxic. The toxicity due to repeated intraperitoneal doses in the rat is slight. In sufficient concentration, hemolysis of human and rabbit red blood cells is effected.

THE COMPOUND, 2-(β-hydroxyethylaminomethyl) - 1,4 - benzodioxane hydrochloride, abolishes or reverses the pressor action of epinephrine in the dog, rabbit, rat, and cat.

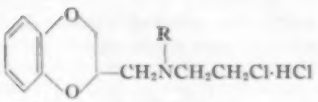
Since Dale (1) first described the blocking and reversal of the action of epinephrine by ergot, many types of compounds have been synthesized and tested for adrenergic blocking activity. Some of the various types studied are: β-haloalkylamines, benzodioxanes, coumarans, ergot alkaloids, imidazolines, yohimbine, and phenoxyethylamines.

It is known that the β-haloalkylamines possess high specificity and long duration of action while the benzodioxanes and coumarans have low specificity and short duration of action. Foltz (2) conceived the idea that possibly the linkage of the β-haloalkylamine group with either the benzodioxane group or the coumaran group would result in compounds possessing high specificity and long duration of action. Thus, one series of derivatives of 2-aminomethyl-1,4-benzodioxane and another of derivatives of 2-aminomethylcoumaran were prepared (Table I).

After screening all of the compounds for adrenolytic activity using rat and dog blood pressure methods, 2 - (β - hydroxyethylaminomethyl)-1,4-benzodioxane hydrochloride (No. 4, Table I) was found to be the most promising. The results of a pharmacological study of this compound constitute the basis of this report.

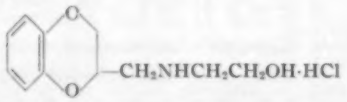
TABLE I.—COMPOUNDS SCREENED FOR ADRENOLYTIC ACTIVITY

1,4-Benzodioxane Series

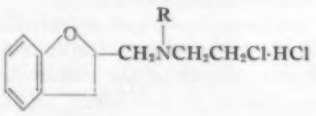


R

(1) methyl
(2) n-amyl
(3) benzyl

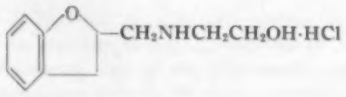
(4) 

Coumaran Series



R

(5) hydrogen
(6) methyl
(7) benzyl

(8) 

EXPERIMENTAL

In each study, except where indicated, 10 to 15 animals were investigated.

Blood Pressure Studies

Anesthetized Rats.—Albino rats, either sex, weighing 175–300 Gm., were anesthetized with 90 mg./Kg. of Amytal® sodium, intraperitoneally. The animals were prepared for recording of the blood pressure in the usual manner. The effect of vagotomy was also observed.

Ten mg./Kg. of the compound, intravenously, produced vasodepression and reversal of epinephrine (1.0 μg./Kg.). The reversal activity lasted for one to two hours. Twenty mg./Kg. of the com-

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pound, when administered intraperitoneally, did not produce vasodepression but reversed the pressor response of epinephrine. Double vagotomy had no influence on the action of the compound. With repeated intravenous injections of 50 mg./Kg. of the compound there was evidence of tachyphylaxis of the vasodepressor response which was quite marked after the third dose. However, the extent of epinephrine reversal was not modified even after repeated injection of the compound. Epinephrine reversal activity was obtained when 150 mg./Kg. of the compound was administered orally and lasted for two to three hours. Vasodepression was not observed after oral administration.

Pithed Rats.—Albino rats, either sex, weighing 200–250 Gm. were anesthetized with 90 mg./Kg. of Amytal® sodium, intraperitoneally. The trachea was isolated and cannulated for connection to a respirator described by Edwards and Miya (3). The rats were pithed according to the method of Shipley and Tilden (4) and were prepared for recording of the blood pressure in the usual manner.

Although epinephrine reversal was not observed in the pithed rat because of the low blood pressure, 10 mg./Kg., intravenously, of the compound caused a slight lowering of the existing low blood pressure and greatly reduced the pressor response of epinephrine (1.0 μg./Kg.). Complete blockage of this dose of epinephrine was not obtained.

Unanesthetized Rats.—The blood pressure of six albino female rats was observed on the photoelectric-tensometer.[1] After taking the normal blood pressure, 100 mg./Kg. of the compound was administered, intraperitoneally. Readings were taken one-half, one, two, and three hours later.

The compound did not exhibit vasodepression. It was not possible to test for epinephrine reversal activity by this method.

Dogs.—Six mongrel dogs were anesthetized with 30 mg./Kg. of pentobarbital sodium, intraperitoneally. The animals were prepared for recording of the blood pressure in the usual manner. Three dogs received 1.0 mg./Kg. of atropine sulfate, intravenously.

Ten mg./Kg. of the compound, intravenously, produced vasodepression and reversal of the pressor response of 5.0 μg./Kg. of epinephrine. In one dog 5.0 mg./Kg. of the compound produced this effect (Fig. 1). Atropine had no influence on the activity of the compound.

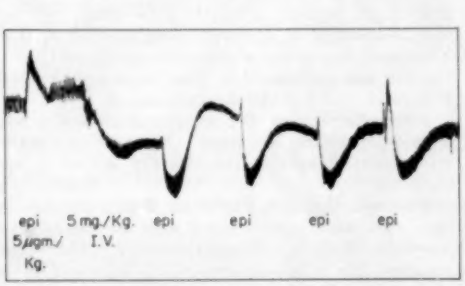

Fig. 1.—Reversal of epinephrine pressor activity by 2(-hydroxyethylaminomethyl)-1,4-benzodioxane hydrochloride on the dog.

[1] Photoelectric-Tensometer Model FAD-1, Metro Industries, Chatham Phenix Bldg., Long Island City, N. Y.

Rabbits.—Rabbits were anesthetized with 1.5 Gm./Kg. of urethan, intravenously. The animals were prepared for recording of the blood pressure in the usual manner.

Ten mg./Kg. of the compound, intravenously, produced only a transient vasodepression. The pressor response of 2.5 μg./Kg. of epinephrine was blocked in all of the five animals employed.

Chickens.—Four white leghorn roosters were anesthetized with 200 mg./Kg. of phenobarbital sodium, intramuscularly. The birds were prepared for recording of the blood pressure by the method of Coon (5).

Thirty mg./Kg., intravenously, of the compound had no vasodepressor effect. Reversal of epinephrine (5.0 μg./Kg.) was not obtained but the pressor response was reduced.

Cats.—Cats were anesthetized with 1 cc./Kg. of a pentobarbital sodium-urethan mixture (3% pentobarb. Na, 25% urethan), intraperitoneally. The recording of the blood pressure and retractions of the nictitating membrane were made in the usual manner.

The compound (5.0 mg./Kg.) produced respiratory arrest when given intravenously. In some cases artificial respiration was beneficial in resuscitation. To overcome this toxicity, 2 mg./Kg. of the compound was administered intraperitoneally. After approximately thirty minutes, intravenous injections of 5 mg./Kg. were tolerated without toxic symptoms. Five mg./Kg., intravenously (7.0 mg./Kg. total dose), of the compound produced vasodepression and blockade of the pressor response of 5 μg./Kg. of epinephrine. This dose also blocked the retraction of the nictitating membrane by epinephrine but did not block the retraction by electrical stimulation. The 2 mg./Kg. dose, intraperitoneally, caused vasodepression. The compound itself produced a prolonged retraction of the nictitating membrane.

Rat Hind Leg Perfusion.—Female albino rats weighing 150–175 Gm. were anesthetized with 90 mg./Kg. of Amytal® sodium, intraperitoneally. A modification of Hyman's (6) method was employed. Aerated Locke-Ringer's solution, U. S. P. was used as the perfusion fluid. A constant pressure of 30 mm. Hg was applied by use of an air "blow-off" system. A constant temperature of 37.5–38° was maintained. Ten to fifteen minutes were allowed for establishment of a constant rate of flow before addition of any drugs, which were injected just proximal to the cannula. The rate of flow was recorded with a drop counter described by Haley and Edwards (7).

Two micrograms of epinephrine (0.2 cc.) caused a 62.1% decrease in flow. Ten milligrams of the compound (0.2 cc.) had no significant effect on the perfusion rate. However, this dose of the compound blocked the vasoconstrictor action of epinephrine. Epinephrine administered simultaneously with the compound produced only a 2.3% decrease in flow. Control injections of Locke-Ringer's solution (0.2 and 0.4 cc.) had no significant action on the rate of flow. A minimum of ten preparations for each dosage level was employed.

Perfused Hearts

Mammalian Hearts (Langendorff's Method).—The hearts of albino rats (approx. 300 Gm.), guinea

pigs (approx. 400 Gm.), and albino rabbits (approx. 2.5 Kg.) were perfused with oxygenated Locke-Ringer's solution, U. S. P. (37.5–38°) at a pressure of approximately 60 mm. Hg. The perfusion fluid was prepared with glass-double distilled water.

A negative inotropic effect was observed in all hearts given doses of 0.5 and 1.0 mg. of the compound. No significant change in heart rate was observed. All hearts returned to normal. On rat hearts, a 13% increase in coronary flow was observed the first minute after the injection of 1.0 mg. of the compound. A 28% increase in coronary flow was observed in guinea-pig hearts while the compound had no significant action on the coronary flow of rabbit hearts. One mg. of the compound blocked the positive inotropic effect of 0.5 μg. of epinephrine on guinea-pig hearts and gave inconsistent blocking on rat hearts. Control injections of Locke-Ringer's solution had no significant action on the hearts. Ten preparations at each dosage level were employed.

Turtle Hearts.—The pulmonary vein was isolated and cannulated with a Greene cannula *in situ*. Sollman and Chu's (8) turtle ringer was used as the perfusion fluid.

Five milligrams of the compound produced a temporary cessation of beat which was followed by recovery of contractility and a decided increase in tone. All hearts gradually returned to normal.

Intestinal Studies (*in vitro*).—Ileal segments of nonfasted albino rats, guinea pigs, and rabbits were used. The segments were suspended in aerated Locke-Ringer's solution, U. S. P. which was maintained at a temperature of 37.5 ± 0.5°. The volume of the bath was 75 cc. throughout the experiment.

A dose of 2.5 mg. of the compound caused a lowering of the tonus of guinea-pig ileum but had little effect on the intestinal movements. Ten milligrams reduced the height of contraction of rabbit ileum while rat ileum was stimulated by 20 mg. of the compound. Ten milligrams showed no antihistaminic effect on the guinea-pig ileum. No spasmolytic effect was observed with this dose against Mecholyl®-induced contractions in the guinea pig, rat, or rabbit ileum. The inhibitory action of epinephrine on rabbit and rat ileum was blocked by 10 mg. of the compound, but no significant effect was observed on the guinea-pig ileum. The blocking action against epinephrine was more easily obtained in the rabbit. Twelve strips from each species for each dose were studied.

Uterine Studies (*in vitro*).—Uteri of nongravid albino rats, guinea pigs, and rabbits were used. The uteri were suspended in aerated Locke-Ringer's solution, U. S. P. which was maintained at a temperature of 37.5 ± 0.5°. The volume of the bath was 75 cc. throughout the experiment.

Twenty milligrams of the compound had no significant action on rat uteri and also had no blocking action against epinephrine. On rabbit uteri, 5 mg. caused an increase in tonus while 2.5 mg. blocked the tonus increase produced by epinephrine. A 2.5-mg. dose caused a prolonged contraction of guinea-pig uteri. Blockade of epinephrine was not observed with 2.5 mg. of the compound on guinea-pig uteri. Nine to twelve strips from each species were employed per dosage level.

Irritation Tests.—Irritation tests were performed by instilling 0.2 cc. of the compound (5%) in rabbit eyes and also by injecting the compound (5%) subcutaneously in rabbit ears according to the method of Kuna and Cuchie (9).

No irritation was noted in the eyes and only a slight erythema was observed in the ears.

Toxicity Studies in Mice and Rats

Acute—Intravenous LD_{50}.—White Swiss mice, 20–38 Gm., and albino rats (Sprague-Dawley), 100–150 Gm., were used. All animals were fasted with water *ad libitum* for twenty-four hours prior to use. A fresh 1% solution of compound was used for the mice and a 5% solution for the rats. The animals were observed in individual cages for twenty-four hours; mortalities counted and LD_{50} determined by deBeer's (10) method.

All mortalities occurred within five minutes with respiratory failure as the cause of death. Clonic and occasional tonic convulsions were noted. Salivation, hyperexcitability, and micturation were also noted. The LD_{50} values with limits of error were 74 mg./Kg. (94–106%) and 102 mg./Kg. (93–107%) for mice and rats, respectively.

Acute—Intraperitoneal LD_{50}.—White Swiss mice, 17–37 Gm., and albino rats (Sprague-Dawley), 45–100 Gm., were employed. The conditions were the same as for intravenous LD_{50}. A fresh 5% solution was used for both rats and mice.

All mortalities occurred within twenty-four hours. Symptoms were the same as for intravenous LD_{50}. The LD_{50} values with limits of error were 385 mg./Kg. (97–103%) and 355 mg./Kg. (94–106%) for mice and rats, respectively.

Acute—Oral.—Six albino rats weighing 100–150 Gm. were given 1.0 Gm./Kg. of the compound (5% solution) by stomach tube. The animals were observed for seventy-two hours in individual cages. No mortalities were observed.

Chronic.—Albino rats were given 50 mg./Kg., intraperitoneally (reversal dose), of the compound daily for sixty days.

A slight nephrosis was observed upon histological examination. No gross or histopathology was observed in the liver, heart, lungs, spleen, and ileum.[2] All animals had a normal growth rate during the period of treatment.

Miscellaneous

Guinea-Pig Tracheal Chain.—The chain was suspended in aerated Van Dyke-Hastings' solution and maintained at a temperature of 37.5 ± 0.5°. The chain was prepared according to the method of Castillo and deBeer (11). The volume of the bath was 75 cc. throughout the experiment.

Ten milligrams of the compound caused a contraction of the tracheal rings. No significant action was observed against the relaxing action of epinephrine.

Electrocardiograph Study on Rats.—Female albino rats were anesthetized with 90 mg./Kg. of Amytal® sodium, intraperitoneally. The right common iliac vein was isolated for injection of the compound. Tracings were taken one and one-half hours after the administration of the anesthetic. After recording the normal, 10 mg./Kg. of the com-

[2] This examination was made by Dr. L. P. Doyle of the Purdue Agricultural Experiment Station.

pound was administered. Tracings were taken immediately, five, ten, and twenty minutes later. Twenty-five mg./Kg. of the compound was also administered.

The compound did not alter the normal ECG pattern. The heart was slowed approximately 60–100 beats per minute. The heart rate returned to normal in about one hour after the administration of the 25 mg./Kg. dose.

Hemolytic Action.—A 5% suspension of washed red blood cells was prepared from rabbit blood. Normal saline solution was used to wash the blood cells and to prepare the 5% suspension of the cells and also to prepare a 5% solution of the compound. Human red blood cells were also used. Test tubes containing 1.0, 0.8, 0.6, 0.4, and 0.2 ml. of the compound were prepared. Two-tenths milliliter of the red blood cell suspension was added to each tube and sufficient saline was added to make a final volume of 1.2 ml. The tubes were kept at room temperature (27–28.5°) and observed after two hours.

Rabbit red blood cells showed partial hemolysis in the tubes where the concentration of the drug was 2.50 and 3.33%. Complete hemolysis was observed at a concentration of 3.33% and partial hemolysis was observed at 2.50%.

DISCUSSION

The results indicate that 2-(β-hydroxyethylaminomethyl)-1,4-benzodioxane hydrochloride acts as an adrenergic blocking agent, orally and parenterally. However, it does not block electrically induced retractions of the nictitating membrane in the cat.

The action of the compound is of a short duration, thereby resembling the benzodioxanes. A compound with a more prolonged action might be obtained by making certain derivatives (ester, ether, tertiary amine) of this compound.

The β-haloalkylamines are irritant and acidified-alcoholic-propylene glycol mixture which is also irritant is required for solution. The compound of choice has the advantage of being soluble in water and is relatively nonirritating.

Blocking action of the pressor response of epinephrine is quite easily produced. However, larger doses of the compound are needed to produce blockade of epinephrine on isolated organs (ileum, uterus, and hearts). Epinephrine is not blocked on all organs due to difference in sensitivity of the various organs of the compound and epinephrine.

In cats the compound causes respiratory arrest when administered intravenously. To overcome this toxic reaction, 2 mg./Kg. of the compound was administered intraperitoneally and approximately 30 minutes later intravenous injections of 5 mg./Kg. of the compound could be made without toxic manifestations.

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The Potentizing Effect of Antimolding Agents in Syrups*

By OLIVER M. LITTLEJOHN† and WILLIAM J. HUSA

In tests using mycophil agar inoculated with microorganisms from deteriorated syrup, mixtures of esters of *p*-hydroxybenzoic acid showed a potentizing action of 5 to 20 per cent in their preservative effect. For the preservation of syrup, storage tests indicated that a mixture of equal parts of butylparaben and methylparaben was effective at a concentration of 1:4500.

ALTHOUGH MANY SUGGESTIONS have been made in the past as to various methods for the preservation of syrups, none of these seems to be entirely satisfactory. The purpose of this investigation was to study the preservation of Simple Syrup, U. S. P., with special emphasis on the effect of using mixtures of preservatives.

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This paper is based on a dissertation presented to the Graduate Council of the University of Florida by Oliver M. Littlejohn, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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HISTORICAL REVIEW

Lord and Husa (1) tested about 225 substances for inhibition of the growth of molds in a Petri dish method which they described.

Mixtures of Preservatives.—In 1932, Sabalitschka (2) recommended the use of a mixture of 60 per cent ethyl *p*-hydroxybenzoate and 40 per cent propyl *p*-hydroxybenzoate for the protection of foodstuffs against deterioration through bacterial or fungal action. In recent years other investigators have suggested various mixtures of the parabens (*p*-hydroxybenzoic acid esters) for

the preservation of pharmaceuticals. Two of these esters are official in the United States Pharmacopeia XIV (3). They are methylparaben (methyl-*p*-hydroxybenzoate) and propylparaben (*n*-propyl-*p*-hydroxybenzoate). Another ester finding wide use as a preservative is butylparaben (*n*-butyl-*p*-hydroxybenzoate) (4).

Schelhorn (5), in 1950, working with combinations of benzoic and citric acids showed that in the presence of citric acid less benzoic acid was required to suppress growth of microbes than when benzoic acid was used alone.

EXPERIMENTAL

Dissolving of Preservatives in Syrup.—Preservatives were dissolved in syrup, with the aid of heat (80°) when necessary. In some cases in which the preservative was not soluble in the syrup it was dissolved in alcohol or propylene glycol and added to the syrup; the syrup contained 1% of either the alcohol or propylene glycol after the addition.

Preservatives which would dissolve in syrup at room temperature or when heated to 80° in the concentrations found to inhibit growth of microorganisms by Lord and Husa (1) were: aldehyde C-20; bay oil; benzoic acid; butylparaben; cetylpyridinium chloride; cinnamic aldehyde; cinnamon oil; citral; eugenol; indol; lemon grass oil; methyl anthranilate; methylparaben; myrcia oil; origanum oil, cretic; oxyquinoline sulfate; pimento oil; propylparaben; rhodinol; thyme oil, red; thyme oil, white.

The following preservatives dissolved in alcohol and then in cold or hot syrup; aldehyde C-14, aldehyde C-18, cassia oil, citronellol, clove oil, rosanlik, and salicylic acid. Anise oil dissolved in propylene glycol and then in syrup.

Seven preservatives would not dissolve in any of the three solvents used. They were: anethol; benzophenone; benzyl butyrate; carnation flower oil synthetic; citral; dimethyl anthranilate and methyl cinnamate.

General Storage Tests.—The twenty preservatives found to dissolve in syrup were used in Simple Syrup, U. S. P. and diluted simple syrup (42.5% sucrose) in the following storage conditions: (*a*) on an east window sill which allowed the direct sun rays to strike the bottle for several hours a day, (*b*) at an elevated temperature obtained in an oven maintained at about 37°, (*c*) in a refrigerator which had an average temperature of 5°, and (*d*) room temperature, about 30°, in a dark place away from all sources of light. The storage tests were conducted for a period of sixty days.

Results of the experiments showed that the concentration of the preservatives was not sufficient to inhibit the growth of molds in all storage conditions. Two of the preservatives, indol and methyl anthranilate, developed an amber color in the sunlight, and odor tests conducted by twelve students indicated that aldehyde C-20, citral, lemon grass oil, cretic origanum oil, and thyme oil were not suitable for simple syrup because of their odors.

Subsequent tests in both the syrups with higher concentrations of the preservatives indicated that some of them could be used in all storage conditions while others could be used in one or more of the storage conditions. In Simple Syrup, U. S. P., none of the preservatives were good in all storage conditions, but the following preservatives prevented growth of molds in the following concentrations in both dark storage and in the refrigerator: bay oil, at 1:1500; cetylpyridinium chloride, at 1:3750; cinnamic aldehyde at 1:9000; cinnamon oil at 1:3000; myrcia oil, at 1:1750; oxyquinoline sulfate at 1:20,000; and rhodinol at 1:4000. In diluted simple syrup (42.5% sucrose w/v) the following concentrations of preservatives inhibited the growth of molds: benzoic acid at 1:750; cinnamic aldehyde at 1:9000; cinnamon oil at 1:4000; methylparaben at 1:750; and rhodinol at 1:2000.

Potentizing Action of Preservatives.—The potentizing action of preservatives may be defined as the increased effect which occurs when a mixture of two or more preservatives has a greater preservative action than the sum of the effects of the preservatives when used alone. Hereafter, the word "potentize" will be used in accordance with this definition. The study was divided into two series using three preservatives in each series. Series I was butyl, methyl, and propyl parabens while Series II was benzoic, cinnamic, and citric acids. The series were divided into groups which consisted of various percentage mixtures of the preservatives. The Petri dish test as described by Lord and Husa (1) was used. In order to obtain reliable information on the potentizing action of two or more preservatives it was necessary to determine more accurately the concentration which would inhibit the growth of molds; this was accomplished by conducting a series of Petri dish tests at various concentrations. After determining the concentration of an individual preservative needed to prevent growth of molds, series of Petri dish tests were conducted using various concentrations of mixtures of preservatives. All tests were performed in duplicate at three different times. The averages of the three tests are recorded in Table I.

The calculation of potentizing action is based on the following considerations: If preservative A was found to inhibit growth of molds at 1:5000 and preservative B at 1:2000 this indicates that 1 Gm. of preservative A will inhibit growth in 5000 Gm. of syrup and that 1 Gm. of preservative B will inhibit growth in 2000 Gm. of syrup. Thus if 0.5 Gm. of preservative A is added to 2500 Gm. of syrup and 0.5 Gm. of preservative B added to 1000 Gm. of syrup each would inhibit the growth of molds in individual containers. If the two were mixed and the preservatives were additive then 1 Gm. of the mixture would inhibit growth at 3500 Gm. of syrup provided there was no potentizing effect. Suppose the mixture of 0.5 Gm. of preservative A plus 0.5 Gm. of preservative B was found by experiment to inhibit the growth of molds in a concentration of 1:5000. The potentizing effect is calculated by dividing the theoretical amounts into the experimental findings:

$$5000/3500 = 1.43 \text{ or } 43\% \text{ potentizing effect}$$

Data on the potentizing effect of parabens and acids to inhibit growth of molds are given in Table I. All calculations are made according to the following example:

Butylparaben inhibited growth at 1:7830
Methylparaben inhibited growth at 1:1080
Butylparaben 90% and methylparaben 10% inhibited growth at 1:7660

$$\begin{array}{rl} 0.90 \times 7830 & = 7047 \\ 0.10 \times 1080 & = \underline{108} \\ 1.00 & = 7155 \end{array}$$

$$\frac{7660}{7155} = 107.1 \text{ or a } 7.1\% \text{ potentizing effect}$$

The effect of *p*H on the potentizing action of mixtures of preservatives was studied by substituting hydrochloric acid for citric acid and determining the concentration of the mixture required to inhibit the growth of molds. By considering the hydrochloric acid as having no power to inhibit the growth of molds, and applying the above calculations it was found that only about 11 to 13% potentizing effect was obtained when hydrochloric acid was mixed with benzoic or cinnamic acids. This result indicates that citric acid has a potentizing effect beyond that which may be attributed to *p*H alone.

By use of pure strains of microorganisms it was found that the parabens exhibited a potentizing action on a *Penicillium* sp., a *Micrococcus* sp. and an unidentified yeast.

Storage Tests Using Mixtures of Preservatives.—The storage tests using mixtures of preservatives involved the use of the parabens and organic acids which had been tested in the potentizing tests. The tests were conducted in the same manner as the general storage tests outlined earlier in this paper. Nineteen mixtures of various percentage compositions at various concentrations were used. Fourteen of the mixtures were of two preservatives in five different percentage compositions, and the other four contained all three preservatives in various percentage compositions. In Simple Syrup, U. S. P., none of the paraben or acid mixtures which were soluble prevented the growth of molds in all storage conditions. In diluted simple syrup (42.5% sucrose w/v) the following concentrations of paraben mixtures were soluble and prevented the growth of molds: butylparaben 90% and methylparaben 10% at 1:7000; butylparaben 65% and methylparaben 35% at 1:6000; butylparaben and methylparaben 50% each at 1:5000; butylparaben 10% and methylparaben 90% at 1:2000; methylparaben 65% and propylparaben 35% at 1:1750; butylparaben 90% and propylparaben 10% at 1:8000; butylparaben 65% and propylparaben 35% at 1:7500; butylparaben and propylparaben 50% each at 1:6000; butylparaben 35% and propylparaben 65% at 1:6500; butyl, methyl, and propylparabens 33⅓% each at 1:5000; butylparaben 50%, methylparaben 25%, and propylparaben 25% at 1:5000; butylparaben 25%, methylparaben 50%, and propylparaben 25% at 1:4000; and butylparaben 25%, methylparaben 25%, and propylparaben 50% at 1:4000. None of the acid mixtures which were soluble prevented the growth of molds.

DISCUSSION OF RESULTS

General Storage Tests.—In Simple Syrup, U. S. P., benzoic acid failed to preserve in all storage conditions in a concentration of 1:1000; growth was observed in concentrations as high as 1:500, but in this high concentration all the benzoic acid was not dissolved. In diluted simple syrup (42.5% sucrose w/v) growth was noted when benzoic acid was used at 1:1000, but no growth was noted in a concentration of 1:750.

Potentizing Action of Preservatives.—The tests showed that there was a definite potentizing action among the parabens, but that the increase in power was not very high. The highest percentage increase was 20.9 using a mixture of 33⅓% of each of the methyl, butyl, and propyl esters at 1:5330. The per cent increase varied from 5.1 for a mixture of 65% methylparaben and 35% propylparaben to 20.9% for an equal mixture of the three parabens. In using one paraben it is often difficult to get a sufficiently concentrated solution because of the low solubility; by means of a mixture of two or more parabens this difficulty is overcome to some extent since a lower concentration of each is required. These tests verify the findings of previous workers (2, 6, 7) that mixtures of parabens are better inhibitors of microorganisms than the individual parabens alone.

The study showed that there was only a slight potentizing action between benzoic and cinnamic acids, but when citric acid was added there was a large increase in the power of the mixture to inhibit the growth of molds. When the citric acid was mixed with either benzoic or cinnamic acid potentizing effects ranging from 20.4 to 83.9% in various

TABLE I.—PER CENT POTENTIZING EFFECT OF MIXTURES OF PRESERVATIVES

Percentage of Mixtures	Mixture A	Mixture B	Mixture C	Mixture D	Mixture E	Mixture F	Mixture G	Mixture H
90–10	7.1	12.5	11.4	...	1.5	31.6	29.4	...
65–35	6.7	5.1	8.6	...	2.5	52.2	38.8	...
50–50	16.0	16.8	12.3	...	1.1	83.9	61.1	...
35–65	16.2	14.6	13.9	...	0.6	83.6	55.5	...
10–90	8.8	12.3	6.9	...	1.6	...	...	...
33⅓ each	...	...	...	20.9	...	...	...	53.3
50–25–25	...	...	...	10.7	...	...	...	40.7
25–50–25	...	...	...	16.5	...	...	...	34.4
25–25–50	...	...	...	13.8	...	...	...	47.7

Mixture A: butylparaben and methylparaben.
Mixture B: methylparaben and propylparaben.
Mixture C: butylparaben and propylparaben.
Mixture D: butylparaben, methylparaben, and propylparaben.
Mixture E: benzoic acid and cinnamic acid.
Mixture F: cinnamic acid and citric acid.
Mixture G: benzoic acid and citric acid.
Mixture H: benzoic acid, cinnamic acid, and citric acid.

mixtures were noted. Mixtures of benzoic, cinnamic, and citric acids showed a potentizing effect of from 34.4 to 53.7% in various percentage mixtures. This study verified the report of Schelhorn (5) that in the presence of citric acid less benzoic acid was required to inhibit the growth of microorganisms than when benzoic acid was used alone. The Petri dish tests indicated that benzoic acid alone would inhibit growth at 1:1030 while an equal mixture of benzoic and citric acids would inhibit at 1:830, or a potentizing effect of 61.1%.

Storage Tests Using Mixtures of Preservatives.—It was found that a mixture of butylparaben 50% and methylparaben 50% at 1:4500 would be best for preservation of Simple Syrup, U. S. P. At this concentration the mixture inhibited growth of molds in both the direct sun rays and the dark storage. At current prices the cost of the parabens for preserving one gallon of Simple Syrup, U. S. P. would be approximately one cent.

In the diluted simple syrup (42.5% sucrose w/v) several mixtures were found to inhibit the growth of molds, but only two could be used in low concentrations. They are butylparaben 90% and propylparaben 10% at 1:8000, and butylparaben 65% and propylparaben 35% at 1:7500. The cost of the parabens for preserving one gallon of diluted simple syrup (42.5% sucrose w/v) would be less than one cent.

SUMMARY

Storage tests indicated that the concentration of the preservatives which had been found to prevent growth in Petri dish tests was not sufficient to prevent growth of molds in four different storage conditions in Simple Syrup, U. S. P. or diluted simple syrup (42.5% sucrose w/v).

Tests showed that there was a definite potentizing action among the parabens, but that the increase was not very great. There was only a slight potentizing action between benzoic and cinnamic acids, but when citric acid was added there was a large potentizing effect.

Storage tests using mixtures of preservatives showed that for Simple Syrup, U. S. P. a mixture of 50% butylparaben and 50% methylparaben at 1:4500 was the best for the inhibition of molds. In diluted simple syrup (42.5% sucrose w/v) the use of either butylparaben 65% and propylparaben 35% at 1:7500, or butylparaben 90% and propylparaben 10% at 1:8000 was effective.

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The Isolation of Three Inositols from *Vernonia Altissima**

By EDWARD J. ROWE, ARTHUR A. HARWOOD, and DONALD B. MYERS

The isolation of *levo*-inositol, *meso*-inositol, and scyllitol from an aqueous extract of the air-dried leaves of *Vernonia altissima* is reported. *Meso*-inositol was isolated from a basic lead acetate precipitate, and *levo*-inositol and scyllitol from the deionized filtrate by fractional crystallization.

Vernonia altissima, Nutt. is one of more than 500 species of *Vernonia* widely distributed in warm-temperature regions (1). It is one of at least eight species (1) growing in the United States, blossoming from July through September. The plant is a composite and is commonly known as "Tall Iron-weed."

The leaves, roots, and seeds of some species of *Vernonia* have been ascribed medicinal properties and some have yielded physiologically active constituents (2–5). However, a literature survey revealed no particular therapeutic application of the *altissima* species nor any chemical study relative to isolation of constituents. An investigation of this plant has been, therefore, undertaken. In this report we present the isolation of three isomeric cyclitols, *meso*-inositol, scyllitol, and *levo*-inositol.

In the course of preliminary selective solvent extractions of the air-dried leaves, it was observed that the alcoholic extract after standing for several months contained a few clusters of well-defined crystals. The crystals were readily soluble in water. Therefore a water extract was prepared and subjected to a general lead process. Based on the weight of the air-dried leaves, yields of 0.08 per cent of *meso*-inositol, 0.2 per cent of scyllitol and 0.4 per cent of *levo*-inositol were obtained.

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EXPERIMENTAL

The leaves of *Vernonia altissima*, Nutt. were collected at the blossoming stage in the environs of Indianapolis, Indiana, and authenticated by the late Dr. Ray C. Friesner of the Botany Department, Butler University. The leaves were air dried and ground in a Wiley Drug Mill through a screen with 2-mm. perforations.

Water Extraction.—Five hundred grams of the ground leaves was placed in a wide-mouthed jar, covered with 3 L. of warm water, and mechanically shaken for three hours. The contents was then transferred to a percolator. Three liters of a chocolate-colored percolate was collected by passing an additional 1500 cc. of warm water through the marc. The percolate was immediately filtered through a pad of pulped filter paper on a Büchner funnel.

Lead Precipitation.—The percolate was treated at room temperature with 400 cc. of a saturated solution of lead acetate. The dense yellow precipitate formed was filtered off, washed, and set aside. The clear yellow filtrate together with the washings was concentrated at 55–60° to 1 L., filtered, and treated with 400 cc. of a 25% solution of lead subacetate. A dense bright yellow precipitate formed. The whole was boiled gently for ten minutes and allowed to cool. The lead subacetate precipitate (A) was filtered off and washed with several portions of hot water. The filtrate (B) therefrom together with the washings totaled 2 L.

Isolation of meso-Inositol.—The lead subacetate precipitate (A) was suspended in 700 cc. of water and treated with hydrogen sulfide. The lead sulfide was removed by filtration and washed with three 200-cc. portions of hot water. The filtrate together with the washings from the lead sulfide was deionized with Amberlites IR-120(H) and IRA-400(OH), and the nearly colorless effluent evaporated at 55–60° to dryness. The tan-colored residue remaining was washed with three 20-cc. portions of cold absolute methanol which removed some of the coloring matter. The washed residue was then taken up with 25 cc. of water, treated with Norit®, and concentrated to 10 cc. Addition of three volumes of ethanol and one-tenth the total volume of ether yielded upon refrigeration colorless, glistening isometric platelets. The yield was 0.08%. The product when recrystallized twice from 70% ethanol melted at 224–225°.[1] There was no depression of the melting point when mixed with authentic *meso*-inositol. A Scherer test for inositols (6) was positive.

Anal.[2]—Calcd. for $C_6H_{12}O_6$: C, 40.00; H, 6.70. Found: C, 40.32; H, 6.94.

The hexaacetate was prepared in the usual manner by the use of acetic anhydride and anhydrous sodium acetate. The crude derivative was purified by several crystallizations from 70% ethanol. The crystals were colorless, pleochromic prisms, somewhat resembling mica. They melted at 215–216° which is in agreement with that recorded for *meso*-inositol hexaacetate.

Anal.—Calcd. for $C_{18}H_{24}O_{12}$: C, 50.00; H, 5.60. Found: C, 50.16; H, 5.58.

Isolation of Scyllitol.—The 2 L. of filtrate (B) and washings from the precipitation of lead subacetate was deionized with Amberlites IR-120(H) and IRA-400(OH). The practically colorless effluent was concentrated at 55–60° to 30 cc. at which volume it was amber in color. An equal volume of boiling ethanol was then added. Granular crystals of scyllitol began to separate out immediately. Separation was considered complete at the end of two days. The crystals were removed from the mother liquor (C). The yield was 1.10 Gm. When crystallized from hot water, glistening, colorless, rhombohedral prisms were obtained which melted at 346–347°[3] with decomposition. Reported melting points for scyllitol 345° (7), 348.5° (8), 352° (9), 353–355° (10).

Anal.—Calcd. for $C_6H_{12}O_6$: C, 40.00; H, 6.70. Found: C, 39.87; H, 6.81.

The hexaacetate was prepared by refluxing the crystalline scyllitol for fifteen hours with acetic anhydride and anhydrous sodium acetate. The crude product when crystallized several times from glacial acetic acid and washed thoroughly with water yielded colorless, glistening, pleochromic prismatic crystals melting at 291–291.5°. Reported melting points for scyllitol hexaacetate: 290–291° (8), 291° (11, 12), 296–297° (7), 299–300° (10), 300° (9).

Anal.—Calcd. for $C_{18}H_{24}O_{12}$: C, 50.00; H, 5.60. Found: C, 49.25; H, 5.54.

Isolation of levo-Inositol.—To the mother liquor (C) which yielded the scyllitol was added one and one-half its volume of hot ethanol. Slow crystallization of *levo*-inositol began after several hours and was considered complete in a week. The mother liquor was decanted and the clusters of crystalline material washed with ethanol and taken up in boiling 70% ethanol. The hot solution was then decanted from a slight amount of insoluble material, refluxed with Norit®, filtered, and allowed to stand for two days at room temperature and then for several more in a refrigerator for crystallization. Seventy-five per cent ethanol appeared to be the most satisfactory solvent for recrystallization. Two grams of crystals were obtained. From ethanol, the crystals were colorless, tetra- and octahedral prisms. The optical rotation was *levo*; $[\alpha]_D^{25} = -65°$ (water). Reported for *levo*-inositol; $-65°$ (13, 14), $-64.1°$ (15, 16), $-63.8°$ (7). The melting point was 246–247°. Reported for *levo*-inositol: 245–250° (16), 247° (15), 249–250° (7). A Scherer test for inositols (6) was positive.

Anal.—Calcd. for $C_6H_{12}O_6$: C, 40.00; H, 6.70. Found: C, 40.68; H, 6.85.

The hexabenzoate was prepared from the crystalline *levo*-inositol using benzoyl chloride and pyridine (16). Recrystallization of the crude product several times from absolute ethanol yielded colorless, pleochromic, prismatic needles melting at 251–252°. Reported for *levo*-inositol hexabenzoate; 252° (13), 253–253.5° (16). The optical rotation was $[\alpha]_D^{25} = -67.7°$ (chloroform). Reported for *levo*-inositol hexabenzoate: $-67.7°$ (16).

Anal.—Calcd. for $C_{48}H_{36}O_{12}$: C, 71.63; H, 4.51. Found: C, 70.94; H, 4.15.

[1] The melting points were determined with a Fisher-Johns apparatus and are uncorrected.

[2] The authors are indebted to Mr. W. L. Brown, Department of Organic Chemistry, Eli Lilly and Company, Indianapolis, Ind., for all of the combustion analyses.

[3] The melting points for scyllitol and scyllitol hexaacetate were determined in capillaries with an Anshütz thermometer.

SUMMARY

1. *Meso*-inositol, scyllitol, and *levo*-inositol have been isolated from an aqueous extract of the leaves of the "Tall Iron-weed."

2. *Meso*-inositol was obtained from the lead subacetate precipitate; scyllitol and *levo*-inositol by fractional crystallization of the deionized and concentrated filtrate therefrom.

3. By weight of air-dried leaves, yields of 0.08 per cent *meso*-inositol, 0.2 per cent scyllitol, and 0.4 per cent *levo*-inositol were obtained.

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The Synthesis and Investigation of Some Ethylene bis-Dithiocarbamate Esters as Fungicides*

By ALFRED P. COLLINS and GAIL A. WIESE

Some esters of ethylene bis-dithiocarbamic acid were synthesized by condensation of disodium ethylene bis-dithiocarbamate with the desired alkyl halide. These compounds were screened for antimycotic activity against five fungi pathogenic to man. A comparison of their activity to that of undecylenic acid was made. The diallyl ester was superior to undecylenic acid in the case of each organism. The bis-2-hydroxyethyl, bis-3-hydroxypropyl and the di-*n*-propyl esters approximated the action of undecylenic acid against several of the organisms.

SULFUR and its inorganic compounds have been recognized for many years as important and useful chemotherapeutic agents for the treatment of fungus diseases of plants, however, in more recent years workers in the field of plant pathology have focused their attention on the study of organic derivatives of sulfur, the first of which were introduced around 1930. Some of the most promising of the numerous organic sulfur compounds that have been studied by plant pathologists are the derivatives of dithiocarbamic acid, i. e., its salts, and the thiuram mono- and disulfides. Because of the intensive investigation that has been given these derivatives as agents for control of plant disease, particularly in view of the broad antimicrobial spectrum they have demonstrated, it is surprising the relatively slight attention they have received from investigators in the field of human chemotherapy.

Investigations since 1931 have shown some derivatives of dithiocarbamic acid to have insecticidal, fungicidal, and disinfecting properties (1–3). The activity of several of these compounds against human pathogenic fungi was first noted in the research laboratories of Bauer and Black in 1938 by Hall. Beginning in 1943 studies on dithiocarbamic acid and other thio-derivatives by Miller and Elson (4) revealed that with each type of derivative, dithiocarbamate, thiuram monosulfide, and thiuram disulfide, the lower members, i. e., the N-methyl and N-ethyl compounds, are the most active and the higher alkyl derivatives are relatively inactive. Kligman and Rosenweig (5) have found on an *in vitro* comparison that the dithiocarbamates possess lower LD_{50} and much greater activity in the presence of blood than do the naphthoquinones, some of which are at present used for the treatment of human fungus disease. Patch tests on rabbits and human subjects were also reported and from the data accumulated the most promising compounds were found to be among the dithiocarbamates. Unpublished findings reviewed by Stedman (6) confirmed in part the data of Kligman and Rosenweig regarding *in vitro* activity of some of the dithiocarbamates but revealed serious disadvantages to pharmaceutical adaptability. Although Kligman and

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Rosenweig have shown the dithiocarbamates to be stable up to eight weeks of storage, it was found that stability of the disodium ethylene bis-dithiocarbamate in aqueous solution depends on the concentration, the more dilute solutions being the least stable. Horsfall (7) reported on the basis of fungicidal tests that dilute solutions show losses of up to ninety per cent of their toxicity.

The literature reveals that van der Kerk and Klopping (8) have tested the methyl ester of dimethyl dithiocarbamic acid and Davies and Sexton (9) have tested the methyl and several other ester derivatives of the same acid. The methyl ester was reported by both groups to possess relatively low activity. Davies and Sexton using *Fusarium graminearum* and *Penicillium digitatum* as test organisms found the allyl ester to inhibit the growth of these fungi in a 1:62,500 concentration. It should be noted that these data were obtained against organisms not commonly considered pathogenic.

A careful perusal of the literature reveals no reference to the preparation of esters of ethylene bis-dithiocarbamic acid, a compound whose sodium salts have displayed an activity two to four times as great as the activity of the sodium salts of dimethyl dithiocarbamic acid. It was therefore of interest to prepare several esters of ethylene bis-dithiocarbamic acid and to determine their antifungal activity against human pathogenic fungi.

EXPERIMENTAL PART I

The following two-step procedure exemplifies the reaction scheme:

$$H_2N{-}CH_2{-}CH_2NH_2 + CS_2 + NaOH \rightarrow (Na{-}S{-}\overset{\overset{S}{\|}}{C}{-}NH{-}CH_2)_2$$

$$(Na{-}S{-}\overset{\overset{S}{\|}}{C}{-}NH{-}CH_2)_2 + R{-}X \rightarrow (R{-}S{-}\overset{\overset{S}{\|}}{C}{-}NH{-}CH_2)_2$$

Preparation of Disodium Ethylene bis-Dithiocarbamate.—*Procedure I:* A solution of 15 Gm. of ethylenediamine in 50 ml. of water was cooled to 30° and to this was added a solution of 20 Gm. of sodium hydroxide in 90 ml. of water also at 30°. Cooling was continued and 38 Gm. of carbon disulfide was added dropwise while stirring over a period of one hour. The reaction mixture was then stirred vigorously for three hours or until all the carbon disulfide had reacted. At the end of this period the reaction mixture was an orange-red solution of disodium ethylene bis-dithiocarbamate. This solution was used directly for preparation of three esters. *Procedure II:* A solution of 30 Gm. of ethylenediamine in 150 ml. of absolute ethanol was cooled to 30°, to this was added a solution of 40 Gm. of sodium hydroxide in 40 ml. of water also at 30°. Cooling was continued and 80 Gm. of carbon disulfide was added while stirring over a period of thirty to forty minutes. After addition of the carbon disulfide the reaction mixture was stirred for two and one-half hours during which time a thick white precipitate of disodium ethylene bis-dithiocarbamate separated. The precipitate was filtered, washed twice with 125 ml. of absolute ethanol, then with two 125-ml. portions of ether and air dried. This salt was used in the preparation of two of the esters.

Preparation of bis-2-Hydroxyethyl Ethylene bis-Dithiocarbamate.—To 195 ml. of the disodium ethylene bis-dithiocarbamate solution (0.25 mole in solution from Procedure I) kept at 30–40°, 40.25 Gm. (0.5 mole) of ethylene chlorohydrin was added slowly while stirring over a period of thirty minutes. After the addition of all the ethylene chlorohydrin the reaction mixture was stirred for three and one-half hours. The ester was then extracted from the reaction mixture with several 50-ml. portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate and the ether was distilled. There remained a bright-yellow, rather viscous oil. The yellow oil was then triturated rapidly with Skellysolve® "A" until crystallization took place. The ester was purified by recrystallizing from a mixture of water and ethanol. Yield, 42 Gm. (56%); m. p. 71–73°.

Anal.—Calcd. for $C_8H_{16}N_2O_2S_4$: N, 9.39; Found: N, 9.39.

Preparation of bis-3-Hydroxypropyl Ethylene bis-Dithiocarbamate.—To 195 ml. of the disodium ethylene bis-dithiocarbamate solution (0.25 mole in solution from Procedure I) kept at 30–40°, 47.25 Gm. (0.5 mole) of trimethylene chlorohydrin was added slowly while stirring over a period of thirty minutes. After the addition of all the trimethylene chlorohydrin the reaction mixture was stirred for three and one-half hours. The reaction mixture was then cooled to 0° and a very viscous yellow oil separated from the solution. The mother liquor was decanted and the yellow oil dissolved in warm isobutylmethylcarbinol, dried over anhydrous sodium sulfate and filtered. The isobutylmethylcarbinol was extracted from the solution with Skellysolve® "A". The Skellysolve "A" remaining in the ester layer was then removed under vacuum at 27°. The yield of the purified ester was 37 Gm. (45%), a viscous undistillable oil.

Anal.—Calcd. for $C_{10}H_{20}N_2O_2S_4$: N, 8.5; Found: N, 8.3.

Preparation of di-*n*-Propyl Ethylene bis-Dithiocarbamate.—To 195 ml. of the disodium ethylene bis-dithiocarbamate solution (0.25 mole in solution from Procedure I) kept at 30–40°, 61.5 Gm. (0.5 mole) of *n*-propyl bromide was added slowly while stirring over a period of thirty minutes. After the addition of all the *n*-propyl bromide the reaction mixture was stirred for three and one-half hours. The reaction mixture was then cooled to 0° and a yellow crystalline precipitate separated. The precipitate was collected on a filter and purified by recrystallizing from ether. Yield, 38 Gm. (51.4%); m. p. 44–46°.

Anal.—Calcd. for $C_{10}H_{20}N_2S_4$: S, 44.3; Found: S, 43.7.

Preparation of Diallyl Ethylene bis-Dithiocarbamate.—To 45 Gm. (0.176 mole) of disodium ethylene bis-dithiocarbamate (from Procedure II) dissolved in 150 ml. of water, 27 Gm. (0.35 mole) of allyl chloride was added slowly over a period of twenty-five to thirty minutes. The reaction mixture was kept at 30–40° and stirred constantly for one and one-half hours. The ester was then extracted from the reaction mixture with several 50-ml. portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate and filtered. The ether was then removed by evaporation with the last small amount being removed under vacuum at 27°. The ester was a bright-yellow, rather viscous oil with an odor very similar to that of garlic. The yield was 44 Gm. (86%) of the ester.

Anal.—Calcd. for $C_{10}H_{16}N_2S_4$: N, 9.6; Found: N, 9.5.

Preparation of Dibenzyl Ethylene bis-Dithiocarbamate.—To 45 Gm. (0.176 mole) of disodium ethylene bis-dithiocarbamate (from Procedure II) dissolved in 150 ml. of water, 44.5 Gm. (0.35 mole) of benzyl chloride was added slowly over a period of twenty-five to thirty minutes. The reaction mixture was kept at 30–40° and stirred constantly for two hours during which time the ester separated as a light yellow precipitate. The precipitate was filtered and washed with 150 ml. of water. The ester was purified by recrystallizing from a mixture of acetone and water. The yield was 30 Gm. (76.5%) of the ester, m. p. 114–116°.

Anal.—Calcd. for $C_{18}H_{20}N_2S_4$: N, 7.14; Found: N, 7.12.

EXPERIMENTAL PART II

The method used for testing for the presence of antimycotic activity of the esters was similar to the procedure described by Kligman and Rosenweig (5). The culture medium used was Sabouraud's dextrose-agar maintained at a *p*H of 5.6 to prevent bacterial contamination.

Water suspensions of spores of the test organisms indicated in Table I were prepared for inoculation in Petri dishes from seven- to fourteen day Sabouraud's dextrose-agar-slant cultures which had been incubated at 37°. Fifteen milliliters of water was added to the slant culture and the growing colony ground very fine, then suspension of the spores brought about by agitation.

The method of testing consisted of pipetting 20 ml. of the culture medium into a sterile Petri dish and allowing the medium to harden. After solidification of the agar, two-thirds of a milliliter of the water suspension of the spores was streaked on each plate. To test each particular fungicide, sterile filter paper disks, 13 mm. in diameter, were placed in an acetone solution of the fungicide. These disks were picked up with sterile forceps and the excess liquid was drained off by touching the disk to the wall of the container. The disk was then immediately dropped onto the agar surface. After a constant incubation at 37° for seven days the diameter of the zones of inhibition were measured and recorded. A control plate for each series using acetone was included in all the observations. The results of the experiments are recorded in Table I.

Due to easily recognized variation that could occur in the determination of *in vitro* antifungal activity in different laboratories using varying techniques, it was decided to compare the activities of the compounds synthesized with the more generally studied antifungal agent undecylenic acid. In the comparative studies, the same media, organisms, and techniques described above were used so that valid comparative results were obtained.

DISCUSSION

The methods used in the preparation of the bis-dithiocarbamic acid esters for this investigation were quite similar to the method described by J. von Braun (10) for preparation of substituted dithiourethanes.

TABLE I.—WIDTH OF ZONE OF INHIBITION IN MILLIMETERS

Organism	Undecylenic Acid	Diallyl Ester	Dibenzyl Ester	bis-2-Hydroxy-Ethyl Ester	bis-3-Hydroxy-propyl Ester	Di-*n*-Propyl Ester	Acetone
M. audouini	21	40	21	25	26	26	0
	26	38	15	21	15	26	0
	27	37	15	15	13	30	
	28	31	15	17	13	28	0
	Av. 25.4	36.3	16.5	19.5	16 7	27.5	0
A. schoenleinii	52	60	32	44	31	40	0
	46	49	31	33	30	35	0
	47	55	26	45	30	35	0
	Av. 48.3	54.7	26.3	40.7	30.3	36.7	0
M. lanosum	34	63	25	43	41	34	0
	36	50	28	47	36	38	0
	Av. 35	56.5	26.5	45	38.5	36	0
M. gypseum	50	65	21	46	32	44	0
	45	64	21	55	50	52	0
	42	60	30	56	50	48	
	Av. 45.7	63	24	52.3	47.3	48	0
T. rubrum	49	63	28	49	30	40	0
	54	47	25	52	50	40	0
	54	55	17	50	60	41	0
	Av. 52.3	55	23.7	50.3	46.7	40.3	0

The process for the preparation of the diallyl and dibenzyl esters where the pure disodium ethylene bis-dithiocarbamate was used proved to be the best method of synthesis. This was especially true when the ester involved proved to be a viscous oil rather than a solid. One side reaction in the synthesis is the hydrolysis of the alkyl halide to form the corresponding alcohol which is very difficult to separate due to the undistillability of the esters. This side reaction is greater in the synthesis using the solution from Procedure I due to the greater basicity of the starting solution.

The results of the tests show that the diallyl ester in every case exhibited an activity somewhat greater than that of undecylenic acid. The bis-2-hydroxyethyl, bis-3-hydroxypropyl and di-*n*-propyl esters in several cases approximate the action of undecylenic acid and may also be worthy of further tests. There was some evidence of species variation toward the agents employed. This was especially true with the bis-2-hydroxyethyl ester which showed comparatively, a much greater activity against *M. lanosum*, *M. gypseum*, and *T. rubrum* than against the other two organisms. The *M. lanosum* showed a considerably greater resistance to undecylenic acid than to the esters tested. Of these esters, the diallyl showed somewhat less variation of activity against the organisms than did any other of the esters or undecylenic acid.

It should be pointed out that the esters were very viscous, a property which tended to inhibit their diffusion out through the media. It is also important to note the activities of the agents were compared on a weight basis whereas a comparison on a molecular weight basis might seem more logical. Had this means been used the esters of ethylene bis-dithiocarbamic acid would very probably have shown much wider variation in comparison to the undecylenic acid since the molecular weights of the esters are greater by a factor of approximately two.

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Identification of Some Sympathomimetic Amines*

By WILLIAM H. A. FISCHER† and ELMER M. PLEIN

Phenylthioureas, benzamides, benzenesulfonamides, 3,5-dinitrobenzoates, and 2,4-dinitrobenzoates of ten commonly used sympathomimetic amines were prepared and when possible the products crystallized and proved by analysis. Melting points of the pure, crystalline derivatives serve as a means of identification. An improved procedure for the preparation of 2,4-dinitrobenzoic acid from 2,4-dinitrotoluene is presented.

INASMUCH as the identification of organic compounds by means of the preparation of crystalline derivatives having sharp melting points is generally regarded as being preferable to the use of color or precipitation reactions, the present investigation was carried out to provide series of derivatives of some sympathomimetic amines for identification purposes. Series of the more usual amine derivatives such as phenylthioureas, benzamides, and benzenesulfonamides and series of the amine salts of two dinitrosubstituted benzoic acids were prepared. The sympathomimetic amines selected were chosen as those widely used in medical practice for local vasoconstriction, nasal decongestion, bronchodilation, and appetite control.

In general, the sympathomimetic amines are identified by color and/or precipitation reactions (1-9). A literature search revealed the preparation of only a few derivatives in the selected series: N-benzoyl amphetamine, m. p. 131–135° (10); N-benzoyl dextroamphetamine, m. p. 160° (11); N-benzoyl phenylpropanolamine, m. p. 143° (12); N-benzoyl ephedrine, m. p. 113° (13); and ephedrine phenylthiourea, m. p. 115° (14). Of these derivatives the first one and the last two were not supported by analytical data.

EXPERIMENTAL

Preparation of Free Amine Bases.—Inasmuch as it was necessary to use free amine bases in the preparation of some of the derivatives, these bases

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were obtained from the salts by treating solutions of them with an alkali and where necessary extracting the base with ether and evaporating the ether solution on a steam bath (2, 4, 7).

All attempts to work with isopropylarterenol were thwarted by the great ease of oxidation of the catechol residue, even in weakly alkaline solution.

Preparation of Phenylthioureas.—Guided by the directions of Shriner and Fuson (15) and McElvain (19) equimolecular quantities of the free base and phenylisothiocyanate were mixed in a test tube. If the free base was a liquid a few minutes' shaking served to complete the reaction. If the base was a solid, the test tube was fitted with an air condenser and the reaction mixture was heated in a water bath for thirty minutes. After cooling, the product was pulverized and washed on a Hirsh funnel with petroleum benzin to remove unreacted phenylisothiocyanate, followed by crystallization from alcohol or water-alcohol mixture.

Preparation of Benzamides.—Following the procedures of Shriner and Fuson (16) and McElvain (20), an amount of amine salt sufficient to yield one gram of the free base was added to 10 ml. of 20% sodium hydroxide solution followed by a 10% excess of benzoyl chloride to cause the Schotten-Bauman reaction. The reaction mixture was cooled in the refrigerator, the solid filtered off and recrystallized from alcohol or water-alcohol mixture.

In the cases of the polyfunctional amines, that is, those containing alcoholic or phenolic groups, no attempts were made to form esters, since it was felt that the presence of polyfunctional groups might not be known in a series of unknown organic bases. Only the N-benzoyl derivatives are reported here.

Preparation of Benzenesulfonamides.—Using the methods of Shriner and Fuson (17) and McElvain (21) as a guide, an amount of amine salt sufficient to yield one gram of base was added to 10 ml. of 10% sodium hydroxide solution and a 10% excess of benzenesulfonyl chloride was added to cause the Hinsberg reaction. The mixture was cooled in the refrigerator, the solid filtered off and recrystallized from alcohol or alcohol-water mixture.

Preparation of the 3,5-Dinitrobenzoates.—In general, the method given by Buehler, Currier, and Lawrence (22) was followed. Equimolecular quantities of the base and the acid were separately dissolved in minimum amounts of boiling absolute alcohol, the two solutions were mixed together and then boiled for a minute before being chilled in the refrigerator. The salts were recrystallized from absolute alcohol, except the salt of 1-methylhexylamine which was purified by dissolving it in dioxane, adding two volumes of water and then chilling in the refrigerator.

Preparation of 2,4-Dinitrobenzoic Acid.—Attempts to prepare 2,4-dinitrobenzoic acid by oxidation of 2,4-dinitrotoluene according to the method of Buehler and Calfee (23) gave very small yields of brownish-red crystals. A few trials showed the superiority of a method in which one and one-half moles of sodium dichromate was placed in 100 ml. of water in a three-necked flask fitted with a thermometer, a mechanical stirrer, and a dropping funnel mounted in a Claissen head to vent the system. One mole of 2,4-dinitrotoluene was suspended in the water and 12 moles of concentrated sulfuric acid was added through the dropping funnel at such a rate that the temperature was held between 30 and 35°, an ice bath being used intermittently as needed. After all the acid had been added, the temperature was allowed to rise spontaneously to 60 to 65°, where it was held by intermittent cooling with the ice bath until the exothermic reaction ceased. The ice bath was then replaced by a heating mantle, and the mixture was held at 60 to 65° for one-half of the time the exothermic reaction had maintained this temperature. After cooling to room temperature, ice was added to double the volume. When the ice had melted, the solids were filtered off and dissolved by divided addition to a saturated solution of sodium bicarbonate. If necessary, more bicarbonate solution was added until the mixture was basic to litmus paper to separate unreacted toluene, which was filtered off. The basic solution was slowly added with vigorous hand stirring to two-thirds its volume of concentrated hydrochloric acid. The acid should not be added to the basic solution, since doing so results in colored crystals, which cannot be decolorized by recrystallization or Norit® treatment. The precipitated 2,4-dinitrobenzoic acid was collected on a Büchner funnel and washed with 200 ml. of cold 6 *N* hydrochloric acid, before being redissolved in saturated sodium bicarbonate solution as above. The precipitation with concentrated hydrochloric acid was repeated. The product was washed with 200 ml. of ice water and dried in a vacuum desiccator over concentrated sulfuric acid. This method yields white crystals, in an amount about 50% of theoretical.

The 2,4-dinitrobenzoic acid was identified by melting point, 183° (18), preparation of the *p*-bromophenacyl ester, m. p. 158° (18), and determination of the neutralization equivalent, 212.12.

Preparation of 2,4-Dinitrobenzoates.—In general, the method given by Buehler and Calfee (23) was followed. Equimolar quantities of the free base and the acid were separately dissolved in the minimum amounts of boiling absolute alcohol, and the two solutions were poured together and boiled for a minute before being chilled in the refrigerator. The salts were recrystallized from absolute alcohol.

DISCUSSION

In a qualitative test such as described here, only pure crystalline products are suitable for melting-point determinations. In several instances oils or pasty substances instead of crystalline materials were obtained. Benzamides of methoxyphenamine and phenylephrine; benzenesulfonamides of 1-methylhexylamine, phenylephrine, and phenylpropylmethylamine; and the 2,4-dinitrobenzoate of phenylephrine did not produce crystalline products and are therefore not included in the table. Also not included in the table are those derivatives which did not analyze for nitrogen within satisfactory limits. All the compounds except the benzamide of amphetamine, the phenylthiourea of methoxyphenamine, the 3,5-dinitrobenzoates of dextroamphetamine and racephedrine, and the 2,4-dinitrobenzoate of ephedrine analyzed within 0.2% of the calculated nitrogen content. These data including the melting points are shown in Table I.

Gadamer (14) reported ephedrine phenylthiourea to melt at 115° but he gave no analytical data for

TABLE I.—MELTING POINTS AND ANALYSES OF SOME SYMPATHOMIMETIC AMINES

Derivatives	Melting Point, °C[a]	Nitrogen Analyses[b] Calcd.	Found
Phenylthiourea of:			
Amphetamine[c]	128–130	10.37	10.30
Dextroamphetamine[d]	104–105	10.37	10.44
Ephedrine[c]	97–98[e]	9.33	9.18
Methoxyphenamine[d]	90–91	8.92	9.28
1-Methylhexylamine[d]	66–67	11.20	11.04
Phenylpropanolamine[c]	170–172	9.79	9.55
Phenylpropylmethylamine[d]	100–101	9.86	9.63
Benzamide of:			
Amphetamine[d]	130–131[f]	5.86	5.47
Dextroamphetamine[d]	159–160[g]	5.86	5.83
Hydroxyamphetamine[c]	139–141	5.49	5.64
1-Methylhexylamine[d]	61–63	6.38	6.30
Phenylpropanolamine[c]	141–143[h]	5.49	5.54
Benzenesulfonamide of:			
Amphetamine[c]	70–73	5.09	5.02
Dextroamphetamine[d]	59–60	5.09	5.10
Ephedrine[d]	94–96	4.59	4.56
Methoxyphenamine[c]	90–93	4.39	4.33
Phenylpropanolamine[d]	124–125	4.82	4.68
Racephedrine[d]	104–105	4.59	4.80
3,5-Dinitrobenzoate[i] of:			
Dextroamphetamine[j]	190–193	12.10	12.44
Ephedrine[j]	194–196	11.14	10.90
Methoxyphenamine[j]	147–148	10.74	10.94
1-Methylhexylamine[k]	133–136	12.84	12.66
Phenylpropanolamine[j]	97–99	11.57	11.68
Phenylpropylmethylamine[j]	187–192	11.63	11.82
Racephedrine[j]	209–212	11.14	11.54
2,4-Dinitrobenzoate[l] of:			
Amphetamine[j]	183–185	12.10	11.88
Ephedrine[j]	189–191	11.14	11.52
Hydroxyamphetamine[j]	190–192	11.57	11.61
Methoxyphenamine[j]	114–116	10.74	10.66
1-Methylhexylamine[j]	128–129	12.84	12.93
Phenylpropanolamine[j]	86–88	11.57	11.42
Racephedrine[j]	166–167	11.14	11.18

[a] Melting points determined with a Fischer-Johns melting point apparatus.
[b] Nitrogen determined in duplicate by micro-Dumas method.
[c] Recrystallized from alcohol.
[d] Recrystallized from alcohol-water mixture.
[e] Gadamer reported 115° (14).
[f] The U. S. P. gives 131–135° (10).
[g] Leithe reported 160° (11).
[h] Hartung, *et al.*, reported 143° (12).
[i] 3,5-Dinitrobenzoic acid melts at 204–205°.
[j] Recrystallized from absolute alcohol.
[k] Recrystallized from dioxane-water mixture.
[l] 2,4-Dinitrobenzoic acid melts at 181–183°.

his product. The compound reported here melted at 97–98° and analyzed within acceptable limits.

Amphetamine benzamide analyzed low in nitrogen (calculated 5.86%, found 5.47%) and melted at 131°. The U. S. P. (10) gives 131–135° as the melting point. Several attempts at preparation and purification did not raise the melting point nor alter the results of nitrogen analysis.

No pure benzamides of secondary amines were obtained. No pure derivatives of phenylephrine or isopropylarterenol were obtained.

The preparation of 3,5-dinitrobenzoates was difficult due to the solubility of both the acid and the salt in absolute alcohol. 2,4-Dinitrobenzoic acid being a stronger acid formed salts which were more easily crystallized.

CONCLUSIONS

1. Attempts were made to prepare five series of eleven common sympathomimetic amines. Melting points of the pure, crystalline products obtained are reported.

2. At least two derivatives suitable for identification were prepared for each of nine amines.

3. An improved method for the preparation of 2,4-dinitrobenzoic acid from 2,4-dinitrotoluene has been developed.

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A Note on Derivatives of *p*-Thymotic Alcohol*

By JOSEPH E. MOODY and PAUL J. JANNKE

***p*-Thymotic alcohol is related structurally to thymol and benzyl alcohol, both of which are accepted therapeutic agents. The preparation of the alcohol and certain of its characterizing derivatives seemed practical in that the compound has theoretical potential as a medicinal substance. The preparation and properties of the alcohol are described and derivatives of it were analyzed and identified.**

IN 1883, KOBEK (1) prepared *p*-thymotic alcohol by reducing the corresponding aldehyde obtained from the reaction between thymol and chloroform in the presence of sodium hydroxide. He reported it to be an amorphous compound melting between 120 and 130°. Lederer (2) prepared the alcohol through the condensation of formaldehyde and thymol in an alkaline medium. The precipitate resulting from acidification of the reaction mixture was fractionally recrystallized from benzene, yielding dithymylmethane (m. p. 149–151°) and *p*-thymotic alcohol (m. p. 116°). Kobek (1), Jensen (3), and Manasse (4) apparently worked with an impure form of the alcohol, since they reported it to produce a deep green color with ferric chloride. Pure thymotic alcohol does not react with ferric chloride, whereas dithymylmethane does, yielding the green color. By working with more concentrated solutions and maintaining a temperature of 15–19° throughout the reaction, Sondern (5) was able to produce the alcohol in 51 per cent yield, with no resinous by-products and no apparent interference from the formation of dithymylmethane.

The purpose of this study was to prepare the alcohol, to determine its physical and chemical properties, and to prepare derivatives of it. In attempting to increase the yield, it was observed that the crude reaction mixture could be freed from unreacted thymol by extraction with petroleum ether, in which *p*-thymotic alcohol is insoluble. The resulting alcohol was readily recrystallized from chloroform.

Derivatives other than those described were repeatedly attempted, but the experiments were not successful. Among them were the phenyl and α-naphthyl urethanes and the sulfonate. Attempts were made to oxidize *p*-thymotic alcohol systematically, however, in each case the ring structure was broken as evidenced by the formation of volatile acids.

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EXPERIMENTAL

Preparation of *p*-Thymotic Alcohol.—Following the improved method described by Sondern (5), 50 ml. of 35% formaldehyde solution (0.6 mole) was added dropwise and with constant stirring to a solution of 90 Gm. (0.6 mole) of thymol in 600 ml. of 5% sodium hydroxide solution. A temperature of 15° was maintained throughout the reaction. The solution was refrigerated for sixteen hours and then filtered to remove a small amount of sediment. It was then acidified fractionally by the addition of 100-cc. portions of 10% acetic acid, removing precipitates as they formed between each addition. The first material which separated was a small quantity of heavy brown oil. It was discarded. The second fraction was a yellow solid mixed with white crystals. Subsequent fractions separated as fine white needles. Each fraction was washed with water until neutral to litmus, and the combined washings yielded additional crystalline matter upon acidification. The crude product, including the impure second fraction, weighed 106.85 Gm. After washing with ligroin to remove unreacted thymol, the alcohol was recrystallized from chloroform. The total yield of pure thymotic alcohol (m. p. 115°) was 62.45 Gm., corresponding to 57.84%.

This procedure was repeated, using larger quantities of reactants, until more than a Kg. was available for further study.

Physical Properties.—*p*-Thymotic alcohol is a colorless, odorless, crystalline compound which is stable in the air when pure, however, when contaminated with phenolic matter it turns yellow and becomes resinous on standing. It melts at 115–116°, and at 2-mm. pressure it sublimes between 85 and 90°. It is readily soluble in low molecular weight alcohols, ether, acetone and hot chloroform. It is poorly soluble in cold chloroform and benzene and only slightly soluble in ligroin and water. The alcohol did not form eutectic mixtures with any of the common eutectifying agents.

Chemical Properties and Derivatives.—*p*-Thymotic alcohol was tested with a number of common reagents to determine whether or not it underwent

characteristic color reactions. Its tests with $FeCl_3$ and with bromine water were negative. The Flückiger test, using NaOH, was negative; however when KOH was used a yellow color resulted. Immediately upon contact with concentrated HNO_3, the alcohol caused violent sputtering, and NO_2 was evolved. Concentrated H_2SO_4 and a mixture of HNO_3 and H_2SO_4 produced a dark red color when applied to the alcohol.

p-Thymotyl Diacetate.—The diacetate of *p*-thymotic alcohol was prepared by two methods, using sodium acetate and pyridine individually as condensing agents, and in each case the reaction product was a pale yellow oil which could not be induced to crystallize. Attempted distillation caused decomposition commencing at 214°. The combined products were distilled under reduced pressure (25 mm.) at 233°, n_D^{25} = 1.4977. The molecular weight of the ester was determined from its saponification equivalent.

Anal.—Calcd. for $C_{15}H_{20}O_4$: mol. wt., 264. Found: mol. wt., 263.88 and 263.08. These results show the compound to be the di-ester of the alcohol.

p-Thymotyl Dibenzoate.—Five Gm. of *p*-thymotic alcohol dissolved in dry pyridine was treated with an excess of benzoyl chloride. The reaction mixture, handled in the usual manner, yielded a yellow crystalline mass which was decolorized and recrystallized repeatedly from ethanol. The white crystalline ester (m. p. 93°) weighed 4 Gm.; yield 40.0%.

The molecular weight of the compound was determined by its saponification equivalent.

Anal.—Calcd. for $C_{25}H_{24}O_4$: mol. wt., 388. Found: mol. wt., 391.6 and 386.0. These results show the compound to be the dibenzoate of *p*-thymotic alcohol.

p-Thymotyl Dinitrobenzoate.—The nitrobenzoate of *p*-thymotic alcohol was prepared in the usual way, beginning with a 5-Gm. sample in pyridine solution to which the *p*-nitrobenzoyl chloride was added. The yellow crystals which resulted were purified by filtering their hot isopropanol solution through charcoal (Norite A). The melting point was constant at 213.5–214.5°. Yield, 2.4 Gm., corresponding to 18.0%.

The percentage of nitrogen in the compound was determined by the Kjeldahl method.

Anal.—Calcd. for $C_{25}H_{22}O_8N_2$: N, 5.9. Found: N, 6.43, and 5.95. This would indicate the compound to be the diester.

Though the nitrogen percentage agrees reasonably well with the theoretical, the compound could not be definitely identified because, contrary to the rules, it was soluble in water and it decomposed sodium carbonate solution. Furthermore, elementary analysis indicated traces of chlorine.

p-Thymotyl Di-3,5-Dinitrobenzoate.—The 3,5-dinitrobenzoate ester of the alcohol was prepared. After purification and recrystallization from acetone-ethanol mixture, 1.8 Gm. of small yellow crystals were obtained, melting at 174–175°.

The percentage of nitrogen in the compound was determined by the Kjeldahl method.

Anal.—Calcd. for $C_{29}H_{20}O_{12}N_4$: N, 9.86. Found: N, 9.58 and 9.95. This showed the compound to be the diester of 3,5 dinitrobenzoic acid.

p-Thymotyl Oxyacetic Acid.—Two Gm. of *p*-thymotic alcohol was treated with 3 Gm. of chloroacetic acid in the presence of 30% NaOH solution. The conditions required for the reaction were met and the acid so produced was purified by extracting an ethereal solution of it with 5% Na_2CO_3 solution. Upon acidifying the carbonate solution, the aryl oxyacetic acid precipitated. It was filtered off and recrystallized from hot water from which it separated in the form of long, fine needles, melting at 95°. The acid weighed 0.4 Gm.

The molecular weight of the acid was calculated from its neutralization equivalent.

Anal.—Calcd. for $C_{13}H_{18}O_4$: mol. wt., 238. Found: mol. wt., 238.7 and 238.52.

Dibromo-p-thymotic Alcohol.—The bromination of 4.0 Gm. of *p*-thymotic alcohol in chloroform with an aqueous solution of potassium bromide and bromine yielded 4.2 Gm. of dark brown oil which could not be crystallized. When distilled at reduced pressure (2 mm.) at a temperature of 135–136°, a yellow oil was obtained.

The percentage of bromine in the compound was determined by the Parr Bomb fusion method.

Anal.—Calcd. for $C_{11}H_{14}O_2Br_2$: Br, 47.33. Found: Br, 47.26 and 47.51. The analysis shows that the dibromo substituent was formed.

Iodo-p-thymotic Alcohol.—A 4.5-Gm. sample of the alcohol was iodinated according to the method suggested by Cook and Martin (6) for the preparation of thymol iodide. The brown liquid which separated from the iodination reaction mixture was dissolved in chloroform, and when ethanol was added a yellow precipitate formed. The material could not be recrystallized, but was reprecipitated from hot ethanolic-chloroform, producing a very pale yellow amorphous compound (0.32 Gm.) melting at 160–162°.

The results of elementary analysis did not agree with the iodine content calculated for any type of compound theoretically formed under these conditions.

SUMMARY

The preparation of *p*-thymotic alcohol and some of its derivatives is described. Among the compounds reported are the diacetate, dibenzoate, dinitrobenzoate, di-3,5-dinitrobenzoate, oxyacetic acid, and the dibromide of the alcohol. An iodine compound is described but its identity is not known. The physical and chemical properties of the alcohol are described briefly.

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Book Notices

Biomathematics. The principles of mathematics for students of biological science. By CEDRIC A. B. SMITH. Charles Griffin & Co. Ltd., London, 1954. xv + 712 pp. 16 x 23 cm. Price 80*s*.

The third edition of *Biomathematics*, thirty years after the first and twenty years after the second edition, attempts to present the fundamentals of mathematics now established as necessary to the studies of biological, medical, and graduate students and researchers. It is not considered to be a textbook on statistics, but a thorough review of the mathematical background necessary to the understanding of statistical applications is included. The scope of the book is best indicated by the titles of the chapters which are: Brush Up Your Arithmetic; Some Points in Algebra; Comparisons of Magnitudes; Shapes and Numbers; Logarithms; Graphical Aids to Calculation; Rates of Change; The Calculation of Small Changes; Relations Involving Rates of Change; Lengths, Areas, and Volumes; Acceleration: Greatest and Least Values; Series; Directed Magnitudes; Some Useful Integrals; Physical and Chemical Magnitudes; Methods of Solving Equations; Matrices; Chance and Probability; Distributions; Simple Statistical Procedures; and Colson Notation: Arithmetic Made Easy.

Useful additions to the book are: a glossary of the principal mathematical symbols, wisely placed at the front of the book; and eight appended tables with explanatory notes and examples of use including logs., sines and statistical tabulations. Answers to problems given in the text are also appended. The type, including the signs and symbols, is clear; the diagrams are sharply drawn; and the author's style of presentation is interesting.

The Lipids. Their Chemistry and Biochemistry. Vol. 2, Biochemistry (Digestion, Absorption, Transport and Storage). By HARRY J. DEUEL, JR. Interscience Publishers, Inc., New York, 1955. xxvi + 919 pp. 16.5 x 24 cm. Price $25.

This second volume of a comprehensive text on the lipids covers the biochemical phase of the subject. Volume I presented the chemistry and chemical classifications of the lipids. It was reviewed in THIS JOURNAL, 41, 57(1952). The recent advances in this field have so greatly increased the amount of information available that the biochemical phase of the subject has been divided into two volumes, the second of which (Volume 3 of the whole set) will include biosynthesis, metabolism, oxidation, and nutritional value of lipids. Volume 2 includes the available information on the digestion, absorption, distribution by blood and lymph systems, and storage of fats and other lipids in the animal body.

Additional chemical information is included in Volume 2. This relates to the properties, composition, and behavior of lipases and lipoxidases; and the chemistry, structure, and composition of the bile acids which must be understood to comprehend fat absorption. The text of Volume 2 is divided into the following chapters: The Digestion and Absorption of Fats in the Gastrointestinal Tract; The Digestibility of Lipids; The Digestion, Absorption, and Digestibility of Lipids other Than Fats; Blood Lipids; The Occurrence of Lipids in the Animal as a Whole; Lipid Distribution in Specific Tissues and in Their Secretions.

Use of this book as a reference source is facilitated by a thorough table of contents and six hundred and thirty references appearing on the pages of citation and listed in an appended alphabetical author index. Also appended is a separate listing of plant and animal sources of lipids in addition to the regular subject index.

The Year Book of Drug Therapy. (1954–1955 Year Book Series.) HARRY BECKMAN, Editor. The Year Book Publishers, Inc., Chicago, 1954. 592 pp. 13.5 x 20 cm. Price $6.

The 1954–1955 volume of this series of reviews edited by Dr. Beckman continues the excellent abstracts of judiciously selected reports from the mass of papers published in the field of clinical medicine. The abstracts are as brief as possible and still give the essential information in the original articles. As usual, the author introduces the text with a very brief review of the outstanding developments during the period covered by the book (journals received between August 1953 and August 1954). This review hits the high spots: starting with the observation that vigorous diuresis resulting from combined use of the salt-free diet and newer diuretic agents does not cause "redigitalization"; it mentions the acceptance of gitalin as a valuable digitalis body; touches on the hypotensive agents with present favor bestowed on rauwolfia and pentolinium; cites the successful oral use of vitamin B_{12} with mucinous materials from hog stomach, but hopes this will not touch off an explosion of oral therapy in pernicious anemia at this premature stage; the successful uses of chlorpromazine are noted; and, in addition to other notable citations, the results of carefully performed studies of the relief from symptoms afforded by placebos precedes the final item in the "thumbnail" sketches which mentions the British commission findings indicating that as good results can be had with aspirin as with cortisone and ACTH in early rheumatoid arthritis. It is interesting to note that as this book is being reviewed, a report released March 8, 1955 indicates that neither cortisone, ACTH, nor aspirin can uniformly end an attack of rheumatic fever in children.

This Year Book series is an excellent reference for pharmacists and physicians.

Cellular Metabolism and Infections. E. RACKER, Editor. Symposium held at the New York Academy of Medicine, March 4 and 5, 1954. Academic Press, Inc., New York, 1954. xi + 196 pp. 16 x 23.5 cm. Price $4.80.

This book records the papers and discussions of the symposium on cellular metabolism and infec-

tions. The material deals with differences in structural and metabolic patterns in hosts and parasites and with metabolic aspects of the infectious process. The scope of the symposium is best indicated by the titles of the papers: Some Singular Features of Bacteria as Dynamic Systems; Some Aspects of the Comparative Biochemistry of Ascaris and of Schistosomes; Energy Production in Animal Tissues and in Microorganisms; Metabolic Adaptation in Animals; Metabolic Approaches to Chemotherapy; Metabolic Factors Affecting Chemotherapeutic Response; Comparative Biochemistry and Chemotherapy; the Tissues and Body Fluids as Media for Pathogenic Agents; Some Effects of Bacteria and their Products on Host-Cell Metabolism; the Role of Host Polysaccharides in Virus Reproduction; Some Metabolic and Cytochemical Aspects of Bacteriophage Infection; Metabolism of Infected Cells; a Study of Phytopathogenic Microbial Toxins of Known Chemical Structure and Mode of Action; Intracellular Survival of Bacteria in Acute and Chronic Tuberculosis; Inhibition of Theiler's GDVII Virus by an Intestinal Mucopolysaccharide; Relation of an Enzyme Dephosphorylating Adenosine Triphosphate to the Virus of Avian Erythromyeloblastic Leukosis; and Alteration of Host Energy Metabolism During Infection.

Of particular interest to pharmacists and pharmacologists are the papers on Metabolic Approaches to Chemotherapy, by A. D. Welch, and Metabolic Factors Affecting Chemotherapeutic Response, by B. D. Davis. Author and subject indexes are appended in this book which should be a useful reference volume in its field.

The National Formulary 1955. By the BRITISH MEDICAL ASSOCIATION and the PHARMACEUTICAL SOCIETY OF GREAT BRITAIN. The Pharmaceutical Press, London, 1955. 210 pp. 11 x 17 cm. Price 5*s* (postage 3*d*), Interleaved 8*s* (postage 5*d*).

The scope, arrangement and style of the 1955 edition of the British National Formulary is similar to that of the 1952 edition which was reviewed in THIS JOURNAL, **41**, 399(1952). The largest groups of the 45 additions are dosage forms of antibiotics and sedatives and hypnotics; while many little-used preparations are included in the 62 deletions. The revision committee concerned itself with nomenclature as is evidenced by 38 changes in titles of drug preparations, particularly injection and tablet dosage forms. But perhaps the most important change is a return to the use of Latin in the principal titles. This change was made to satisfy the complaints of many physicians and pharmacists who use the Formulary in their practices under the British National Health Service.

Calendar of the Pharmaceutical Society of Great Britain 1954/55. The Pharmaceutical Press, London, 1954. vi + 306 pp. 14.5 x 22 cm. Price 12*s*.6*d*. (postage 10*d*).

The annual compilation of the Pharmaceutical Society of Great Britain for 1954–1955 includes general information such as the various officers, committee members, meeting dates, poisons board, statement on professional conduct, and branches, as well as the administration and activities of the various component groups and bodies of the Society. The British pharmacy acts starting with the Act of 1852 are included along with other rules and regulations. The Calendar is of particular interest to pharmacists in the British Commonwealth.

Glutathione. Proceedings of the Symposium held at Ridgefield, Connecticut, November, 1953. Academic Press, Inc., New York, 1954. x + 341 pp. 15.5 x 23.5 cm. Price $7.50.

This book includes a series of papers by outstanding workers in the related fields of chemistry, biology, and clinical aspects in which glutathione is involved. Following the paper and its bibliography are pertinent questions and answers from the discussion of the subject. The 28 papers are divided into four groups under the headings: Properties and Organic Chemistry; The Methods for Detection and Assay of Glutathione and Sulfhydryl Compounds; Biochemical Mechanisms; Physiological Actions and Clinical Aspects. Four of the nine papers in the latter group deal with the role of glutathione in conditions related to radiation effects. The book is illustrated, and has author and subject indexes. It is an excellent reference to the views in this field as of November, 1953, when the symposium on glutathione was held.

Perspectives and Horizons in Microbiology. A Symposium. Edited by SELMAN A. WAKSMAN. Rutgers University Press, New Brunswick, N. J., 1955. x + 220 pp. 14.5 x 22 cm. Price $3.50.

This book is a compilation of the papers read at a symposium in connection with the dedication on June 7, 1954, of the Institute of Microbiology, Rutgers University, and it has a foreword and a statement by the Institute's Director, Selman A. Waksman. The thirteen papers that are included in the symposium are divided into three sections which are headed: the microbe as a living system; metabolism of microorganisms; and microorganisms and higher forms of life. Most of the papers include bibliographies but no general index is given. Of particular immediate interest is the paper "Microorganisms and Steroid Transformations," by Durey H. Peterson. Microorganisms that cause oxygenation of steroids at definite carbon atoms and produce a specified orientation are listed, and diagrammatic equations indicate other induced molecular changes.

Outlines of Enzyme Chemistry. By J. B. NEILANDS and PAUL K. STUMPF. John Wiley & Sons, Inc., New York, 1955. viii + 315 pp. 16 x 23.5 cm. Price $6.50.

This book is intended to introduce senior undergraduates and beginning graduate students who have had thorough grounding in chemistry, physiology, and biochemistry to the general subject of enzyme chemistry and to provide background material for research workers in fields other than enzymology. The language and presentation are accordingly unusually understandable and interesting in a work of this nature. The text is liberally illustrated with tables, equations, and diagrams. The text which is based on lecture material presented in a one-semester course is divided into four parts under the headings: General Principles; Physical Chemistry; Types of Coenzymes and Enzymes; and Metabolic Patterns. Over half the text is devoted to the general properties of enzymes, and

throughout the subject matter is treated in outline form in an attempt to bring into focus the entire field of enzyme chemistry.

The book has an excellent format, and its chapter bibliographies and index aid in making it a desirable reference volume as well as a very good textbook.

Rapid Detection of Cations. By GASTON CHARLOT DENISE BEZIER, and ROLLAND GAUGUIN. Translated by RALPH E. OESPER. Chemical Publishing Co., Inc., New York, 1954. 92 pp. 13.5 x 20 cm. Price $3.

This small book presents in concise form reagents and procedures for identifying cations in prepared sample solutions containing 10,000 to 100–10 parts per million according to the sensitivity of the reaction used. No equations are given. It is a handy reference for advanced students and investigators.

Drugs in Current Use 1955. Edited by WALTER MODELL. Springer Publishing Company, Inc., New York, 1955. ix + 147 pp. 14 x 21 cm. Price $2.

A dictionary-style list of current drugs by generic and trade names includes brief statements on pharmacologic actions and uses, dosage forms, administration, and dose. The usefulness of such a book is limited, as far as the pharmacist is concerned, by omission of the names of manufacturers or suppliers of drug products. The selections for inclusion in the list are based on the requirements of physicians rather than pharmacists but the compilation is a handy reference for all handlers of drug products.

Actualités Pharmacologiques. Series No. 7. RENE HAZARD, Editor. Masson et Cie, Paris-6, France, 1954. 210 pp. 16 x 25 cm. Price 1480 fr.

The seventh volume of this series of compiled papers in the field of basic and applied pharmacology continues the high standard of presentation set earlier. However, although the style of French used is easily read, the editor need not avoid English summaries as in the case of the first article on "Acetylcholine and the Maintenance of Cardiac Rhythm," by J. M. Burn (University of Oxford). Other papers by different authors in this edition are: Ion Exchange Resins and Their Therapeutic Applications; Synthetic Curare and Anticurare Compounds—Mechanism of Action; on the Mechanism of Action of Anti-Inflammatory Substances; Discussion of the Different Activities of the Transformation Products of Procaine; Analgesic Action of Morphine—its Experimental Determination and Pharmacologic Analysis; New Applications of the Concept of Antagonism by Structural Analogy; and Substitutes for Human Plasma—Special Study of Deanaphylactized Animal Plasma.

Catalysis. Vol. 2. Edited by PAUL H. EMMETT. Reinhold Publishing Corporation, New York, 1955. vi + 473 pp. 16 x 23.5 cm. Price $12.

This is one of a series of books designed to provide research chemists with a well-integrated presentation of the physical chemistry of catalysis and catalytic processes. Volume I was devoted to catalyst supporters, carriers, and methods of preparation. Volume II covers fundamental theories, and Part 2 of this volume is divided into six chapters in which heterogeneous catalytic vapor phase reactions are classified and discussed, followed by reaction rates and selectivity in catalyst pores, the nature of catalytic surfaces, nature of complexes on catalyst surfaces, general theories of heterogeneous catalysis, and finally catalysis in homogeneous reactions in a liquid phase.

The book is provided with an author and subject index, and is thoroughly documentated with references to the original literature.

BOOKS RECEIVED

Biomathematics. The principles of mathematics for students of biological science. By CEDRIC A. B. SMITH. Charles Griffin & Co. Ltd., London, 1954. xv + 712 pp. 16 x 23 cm. Price 80*s*.

The Nucleic Acids, Volume I. Edited by ERWIN CHARGAFF and J. N. DAVIDSON. Academic Press Inc., New York, 1955. xi + 692 pp. 15.5 x 23.5 cm. Price $16.80.

Methods of Biochemical Analysis. Vol. 2. Edited by DAVID GLICK. Interscience Publishers, Inc., New York, 1955. vi + 470 pp. 16 x 23.5 cm. Price $9.50.

Analytical Cytology. ROBERT C. MELLORS, Editor. The Blakiston Division, McGraw-Hill Book Company, Inc., New York, 1955. 16 x 23.5 cm. Price $15.

Biochemistry and Physiology of Protozoa. Vol. 2. S. H. HUTNER and ANDRÉ LWOFF, Editors. Academic Press, Inc., New York, 1955. xiii + 388 pp. 16 x 23.5 cm. Price $9.

Pomp and Pestilence. Infectious Disease, its Origins and Conquest. By RONALD HARE. The Philosophical Library, Inc., New York, 1955. 224 pp. 13.5 x 21 cm. Price $5.75.

Rutin and Related Flavonoids. By JOHN Q. GRIFFITH, JR., CHARLES F. KREWSON, and JOSEPH NAGHSKI. Mack Publishing Company, Easton, Pa., 1955. 13 + 275 pp. 16 x 23.5 cm. Price $6.00.

Ion Exchange and Adsorption Agents in Medicine. The Concept of Intestinal Bionomics. By GUSTAV J. MARTIN. Little, Brown and Company Boston, 1955. Illustrated. vii + 333 pp. 16.5 x 24.5 cm. Price $7.50

Substances Naturelles de Synthèse. Préparations et Méthods de Laboratoire. Vol. 10. LÉON VELLUZ, Editor. Masson et Cie, Paris-6^{e}, 1954. 201 pp. 16 x 22.5 cm. Illustrated. Price (paper) 2.300 fr.; (cloth) 2.700 fr.

Les Gaz Inertes, L'Hydrogène—Les Halogènes. By P. LAFITTE and H. BRUSSET. Masson et Cie, Paris-6^{e}, 1955. 396 pp. 17.5 x 25 cm. Price (paper) 3.600 fr.; (cloth) 4.200 fr.

Amphetamine in Clinical Medicine: Actions and Uses. By W. R. BETT, LEONARD H. HOWELLS, and A. D. MACDONALD. The Williams & Wilkins Co., Baltimore, 1955. (E. & S. Livingstone, Ltd., Edinburgh and London). 78 pp. 13 x 19 cm. Price $2.75.

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